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**Propper et al.**

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(54) **ANURAN CROSS-SPECIES MOLECULAR SENSORS**

(2013.01); *C12Q 1/6883* (2013.01); *C12Q 1/6888* (2013.01); *G01N 33/5014* (2013.01); *G01N 33/78* (2013.01); *C12Q 2600/142* (2013.01); *C12Q 2600/158* (2013.01); *C12Q 2600/16* (2013.01); *G01N 2333/4606* (2013.01); *G01N 2520/00* (2013.01)

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(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

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Skirrow et al., *Developmental Dynamics* vol. 237 (2008) pp. 3787-3797.\*

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(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

\* cited by examiner

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(22) Filed: **Oct. 7, 2014**

(65) **Prior Publication Data**

US 2015/0094228 A1 Apr. 2, 2015

#### Related U.S. Application Data

(60) Provisional application No. 61/883,138, filed on Sep. 26, 2013.

(51) **Int. Cl.**

***C12Q 1/68*** (2006.01)

***G01N 33/50*** (2006.01)

***G01N 33/78*** (2006.01)

(52) **U.S. Cl.**

CPC ..... ***G01N 33/5088*** (2013.01); ***C12Q 1/6851***

(57) **ABSTRACT**

Described herein are DNA primer sequences designed for the determination of gene or transcript information from Anuran species, and which may be used in studies for developmental and/or toxicity testing and for environmental toxicology or ecological assessment. Also described herein is a rapid, sensitive, high-throughput assay useful for supporting potential risk assessment across vertebrate clades, and that is also useful for evaluation of complex contaminant mixtures.

**4 Claims, 37 Drawing Sheets**

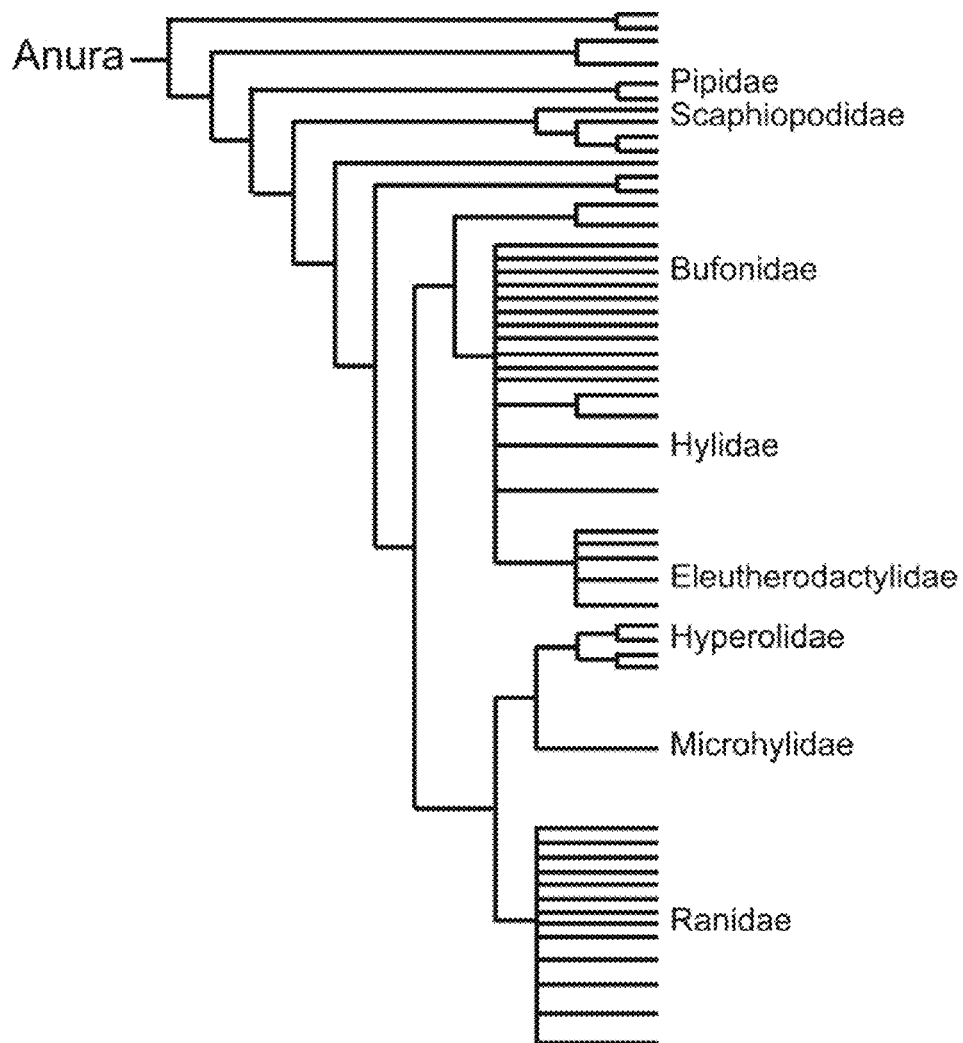
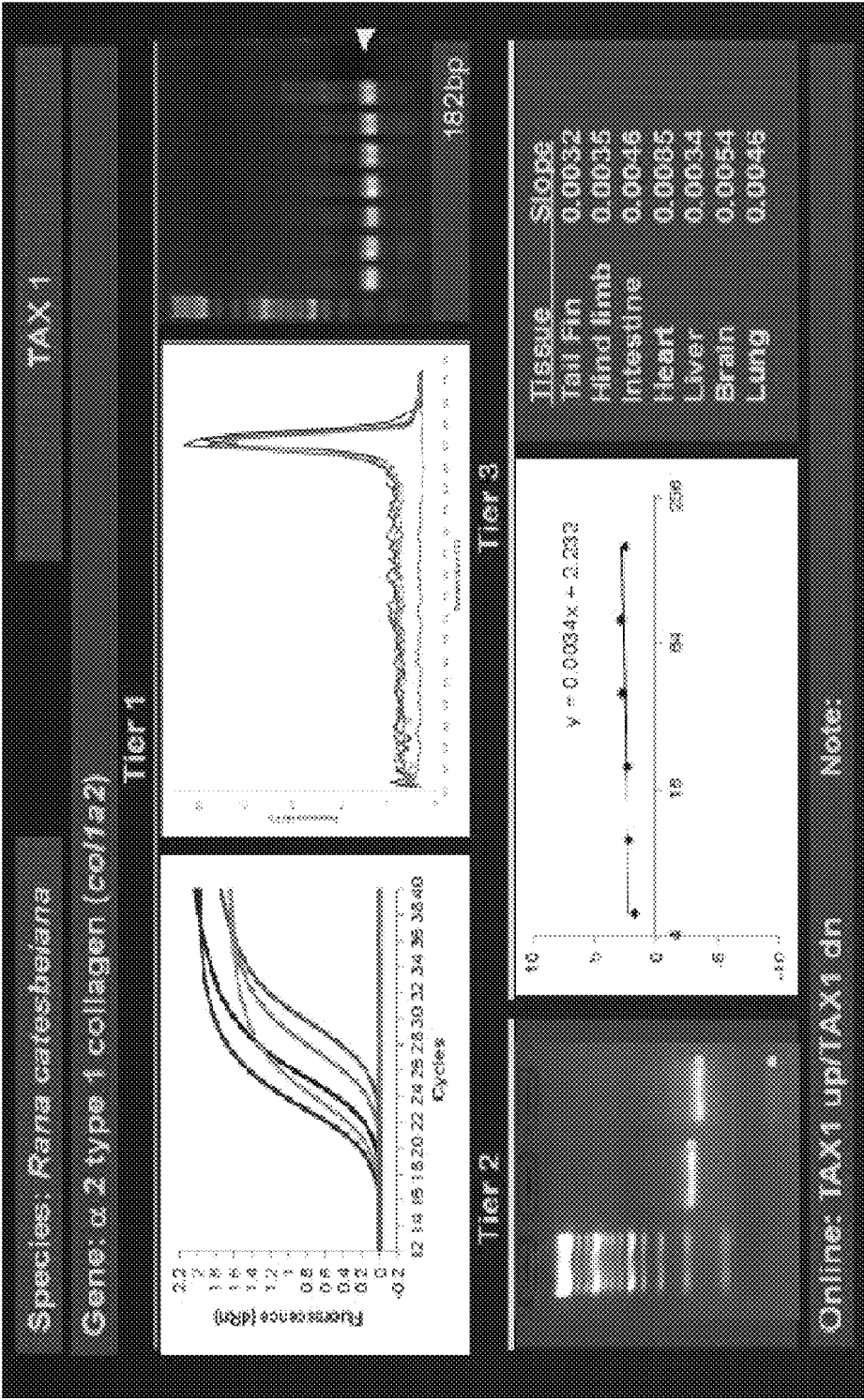


FIG. 1



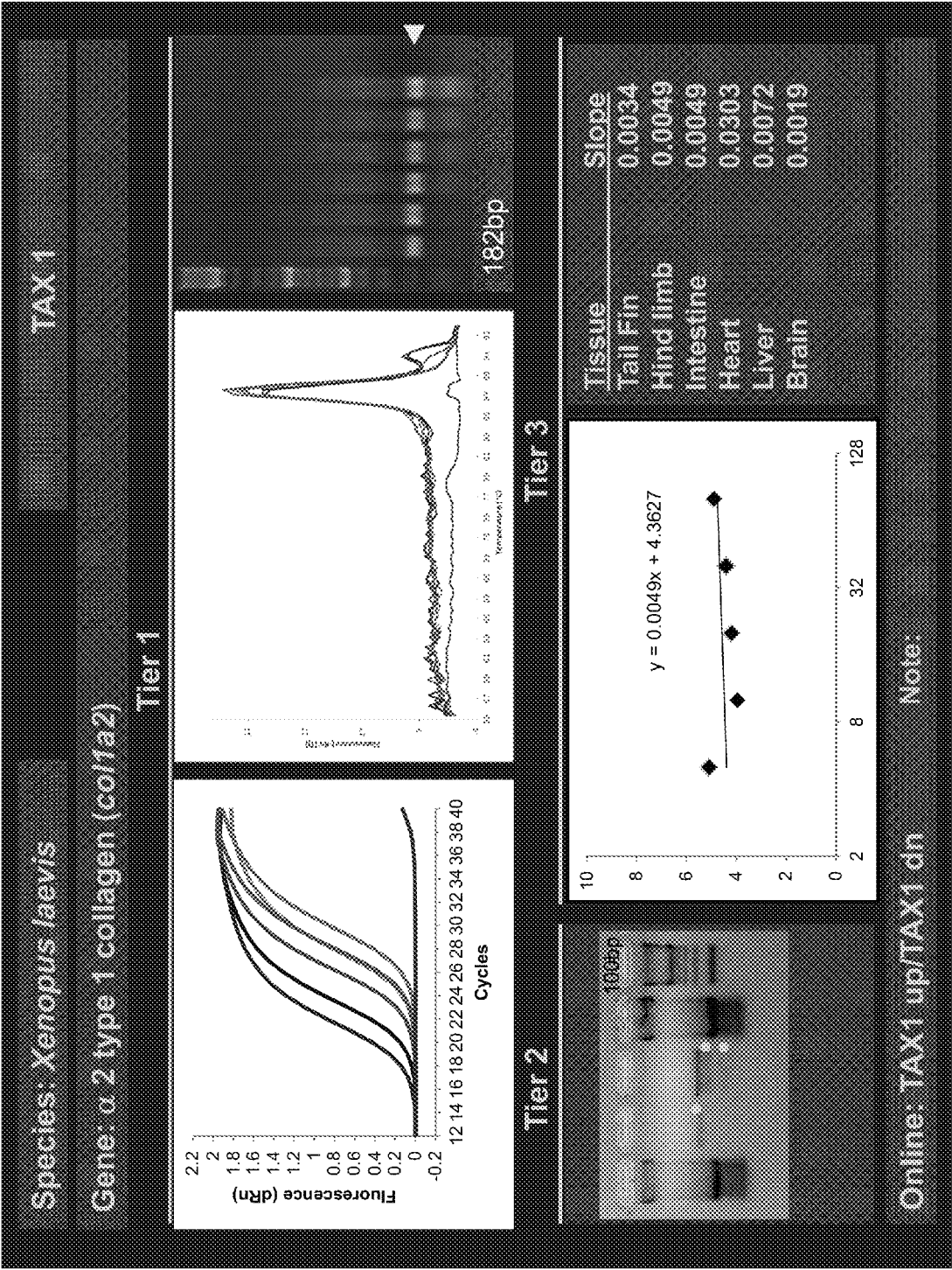


FIG. 2A(2)

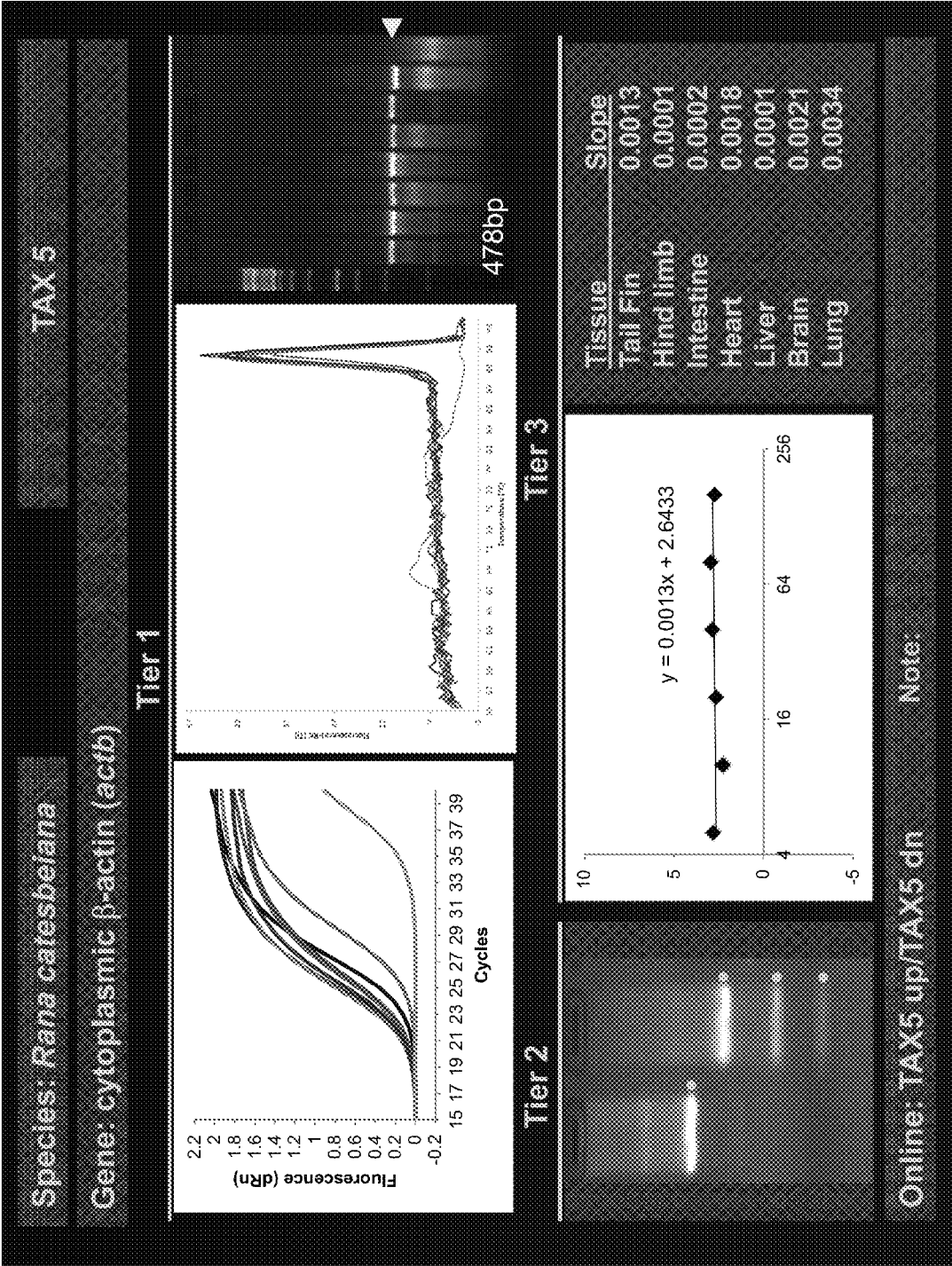


FIG. 2A(3)

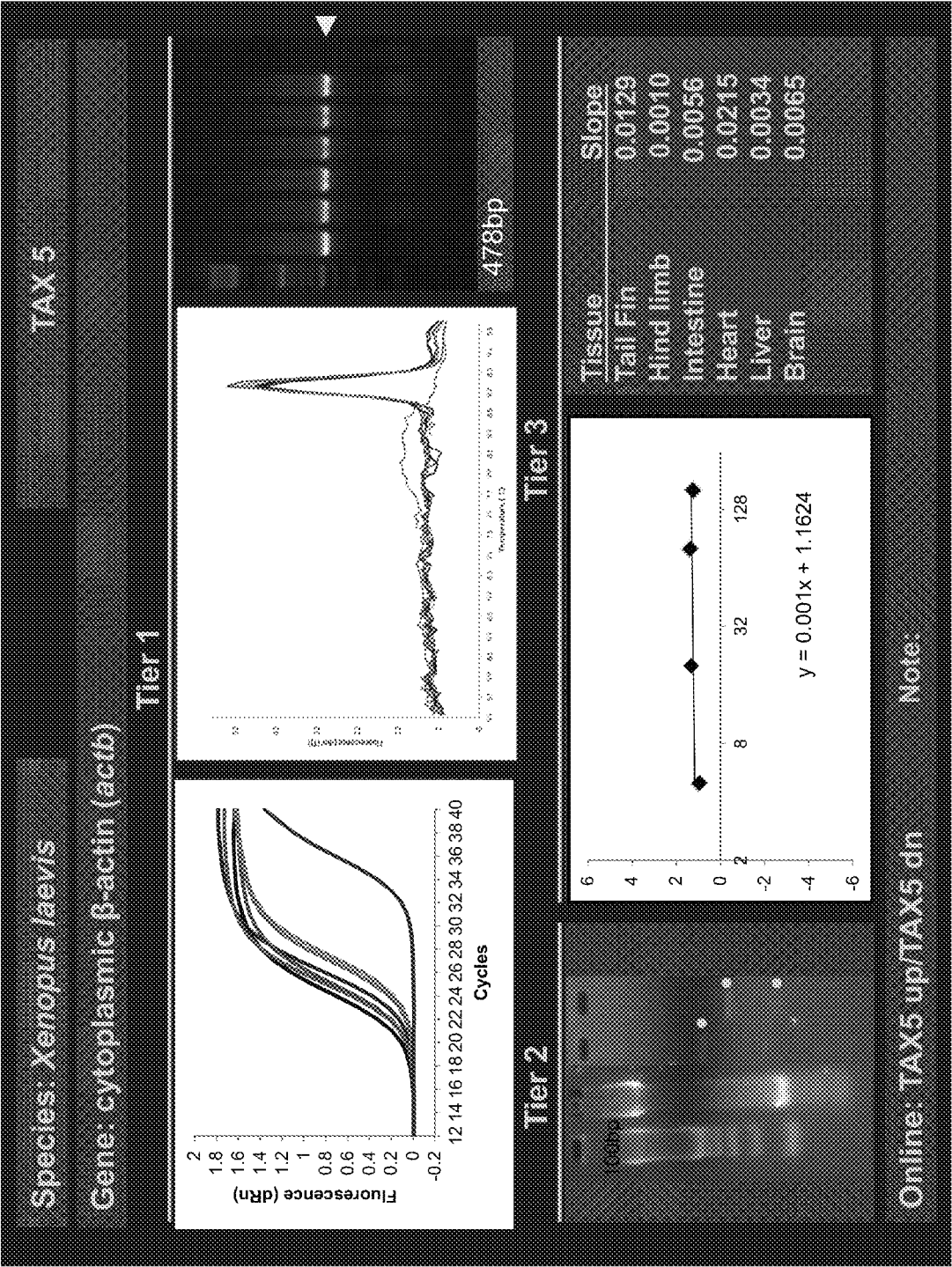


FIG. 2A(4)

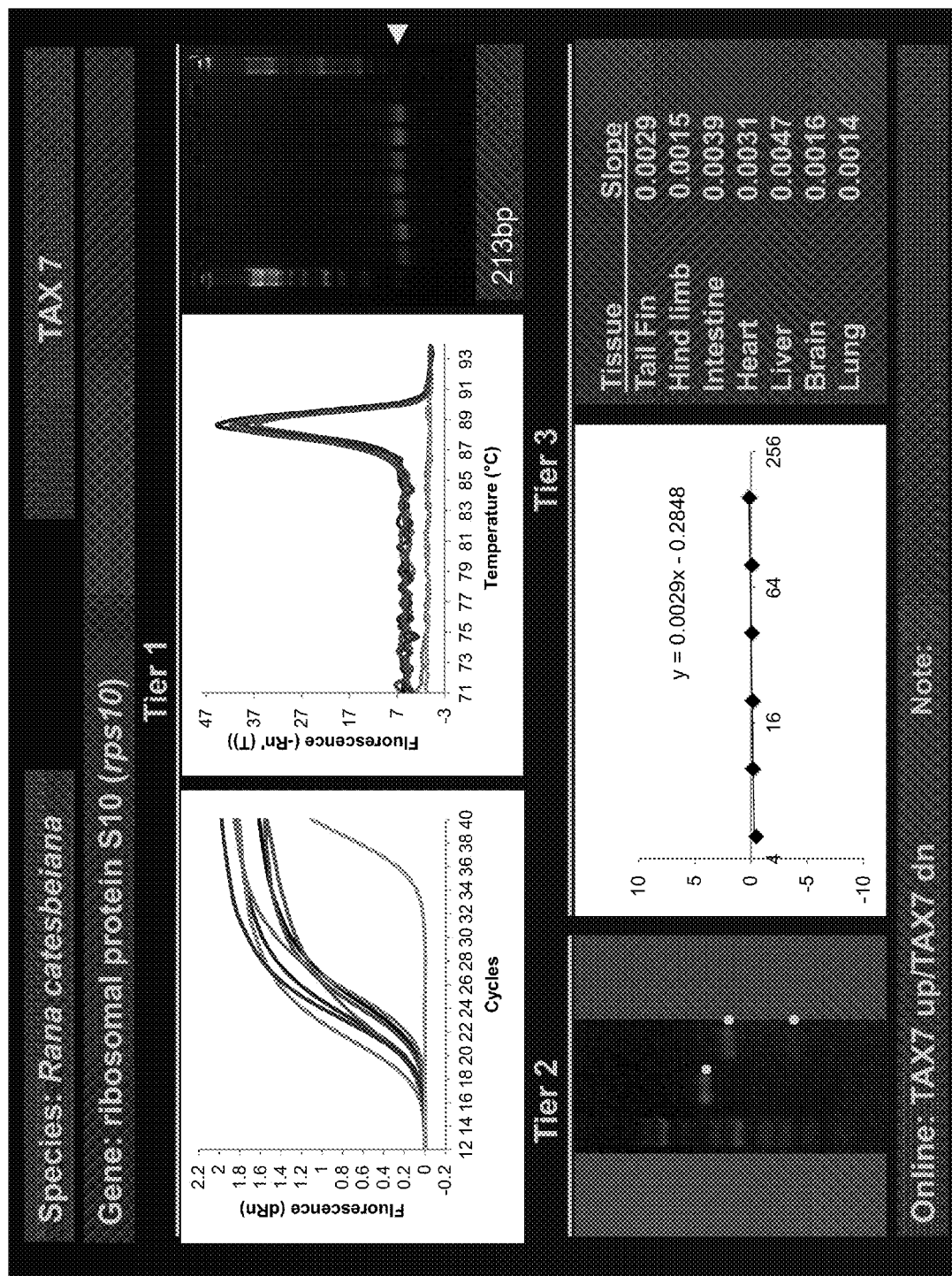
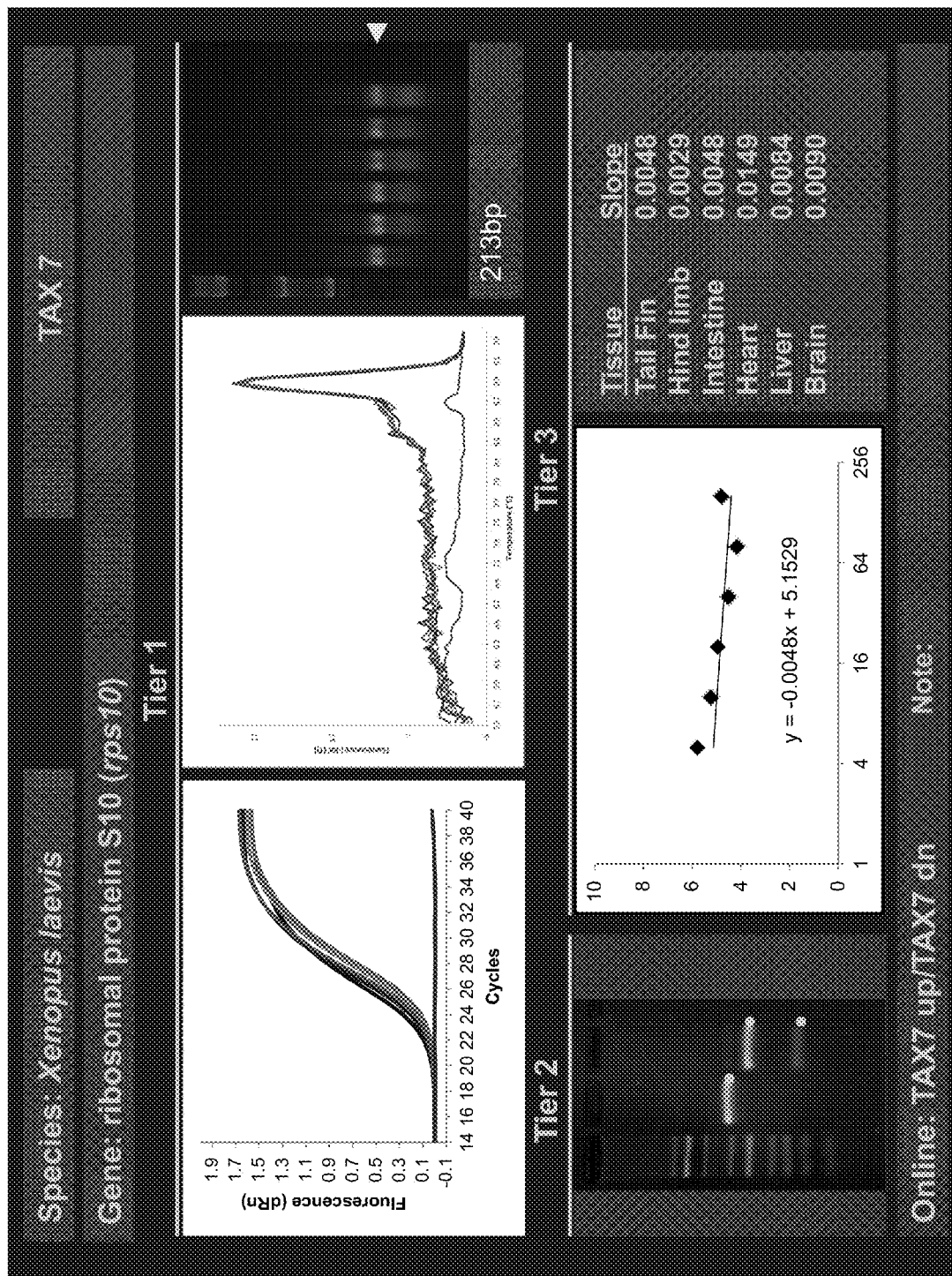
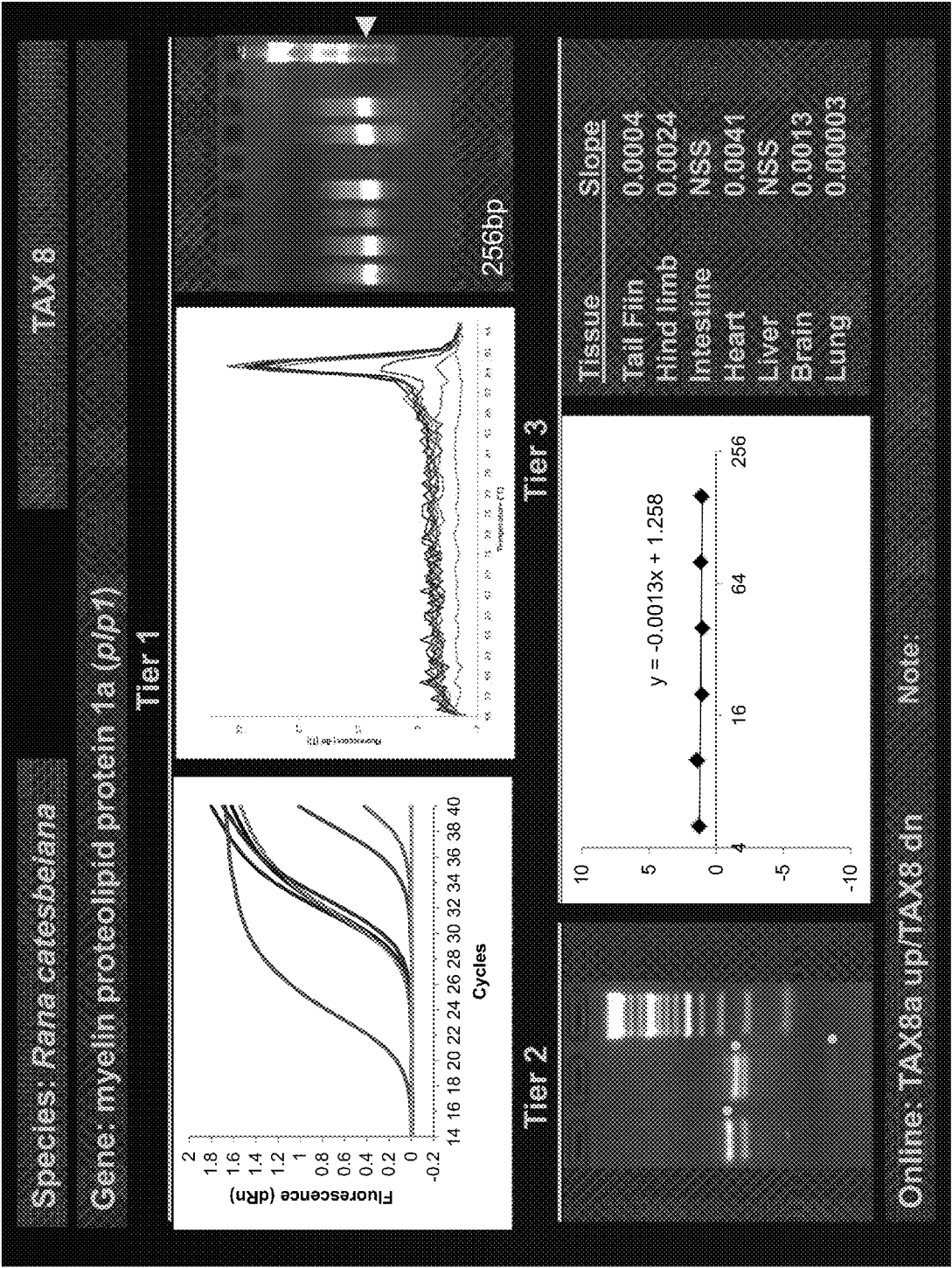


FIG. 2A(5)





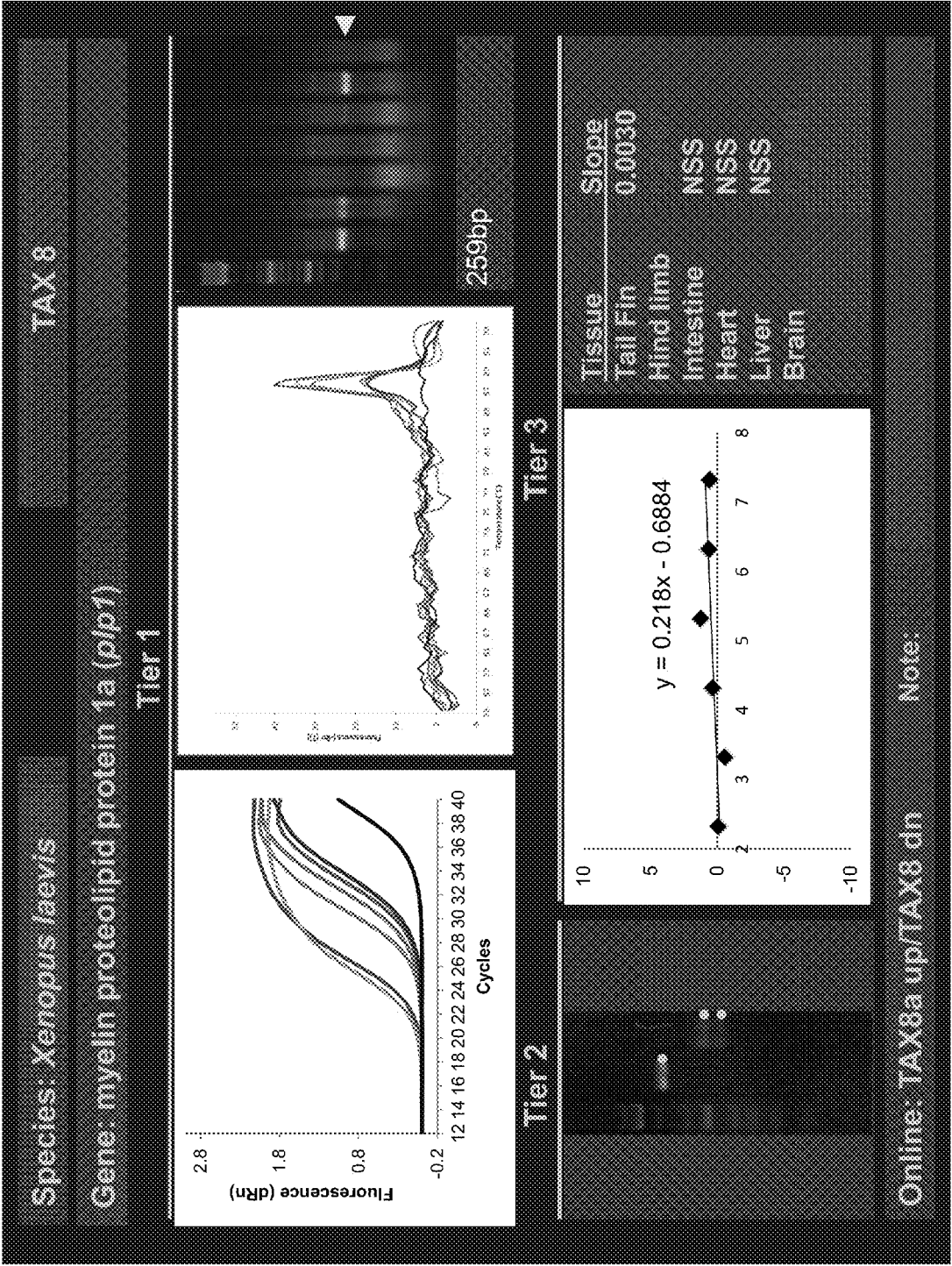


FIG. 2A(8)

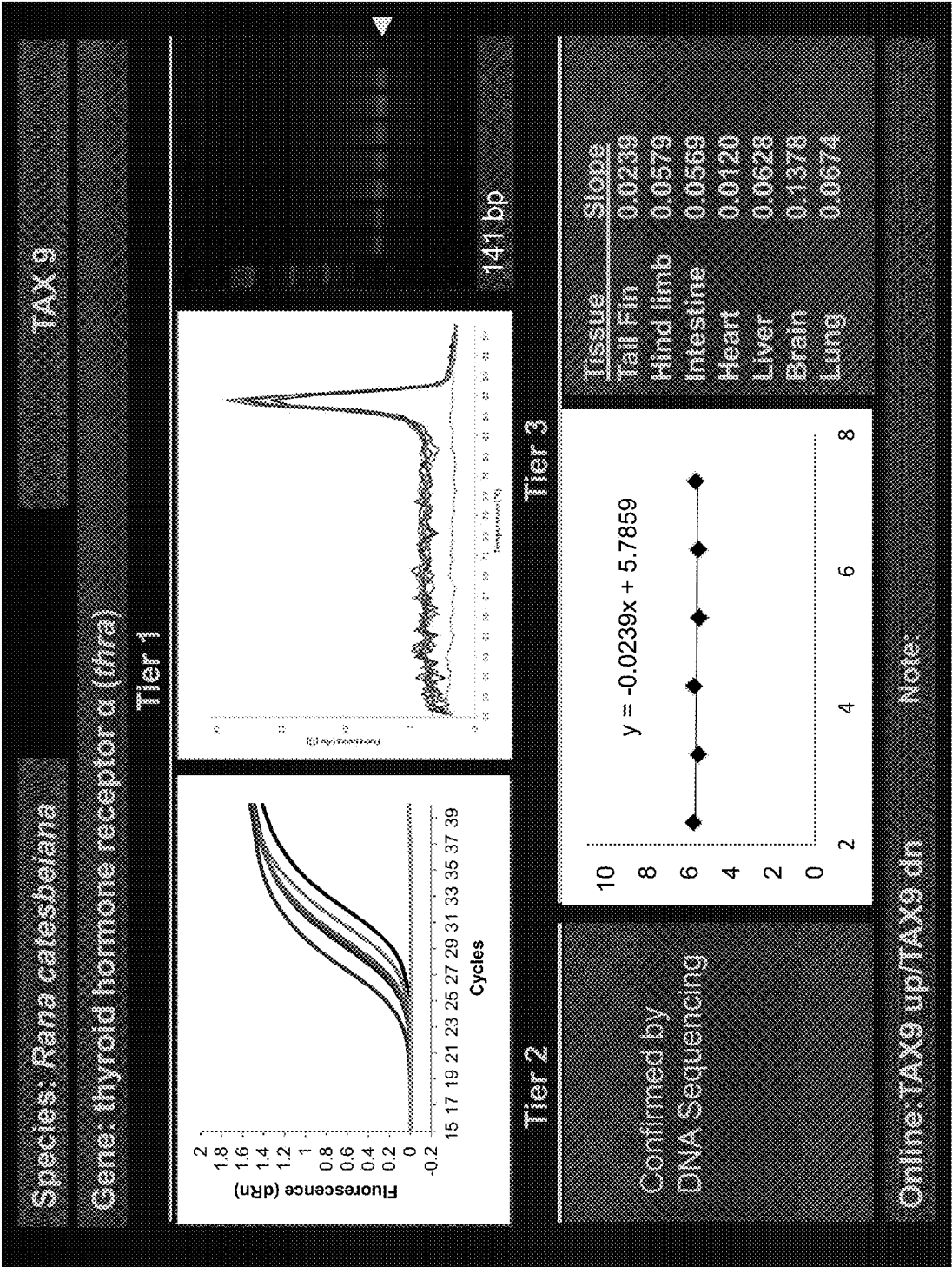
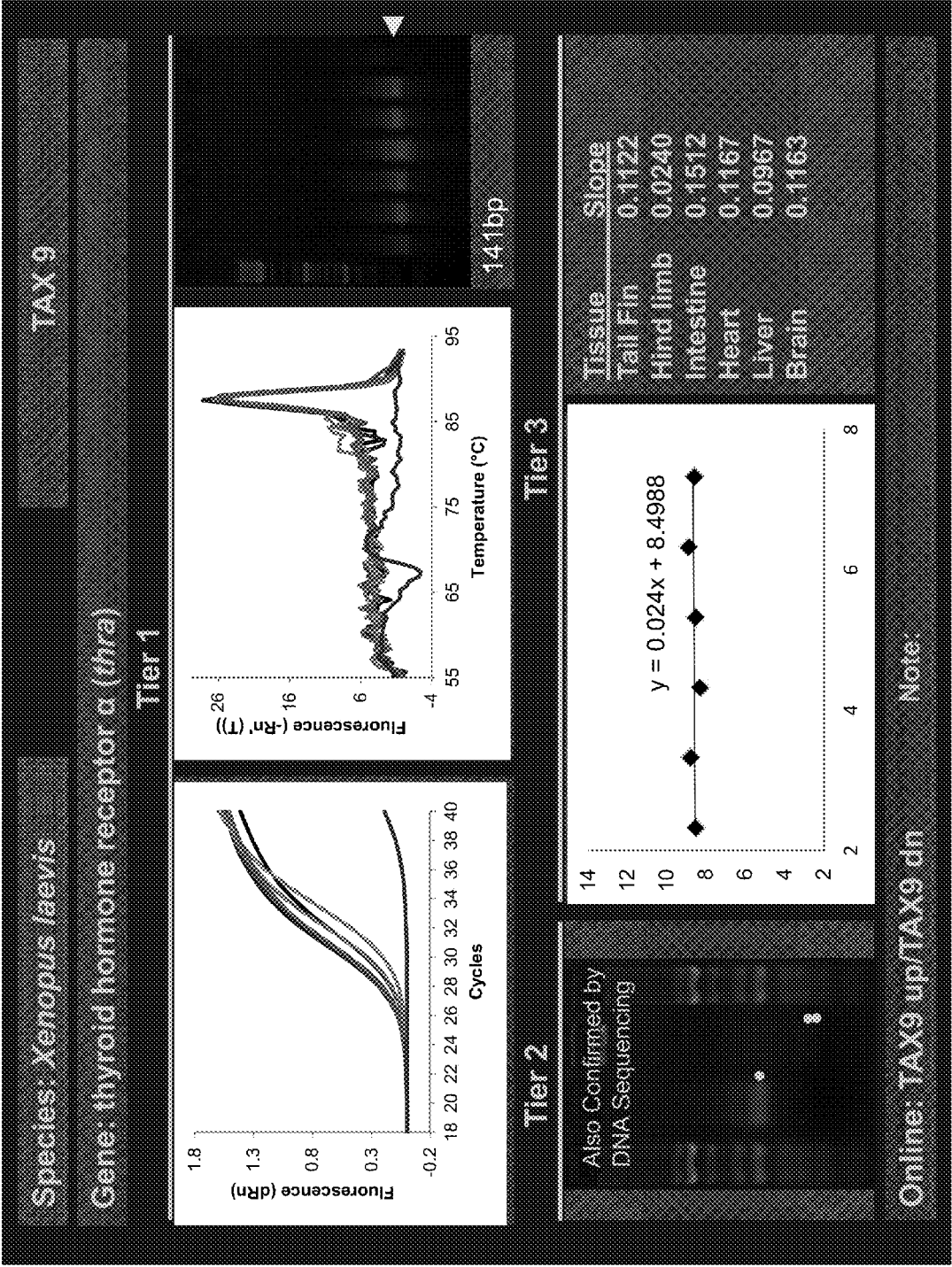


FIG. 2A(9)



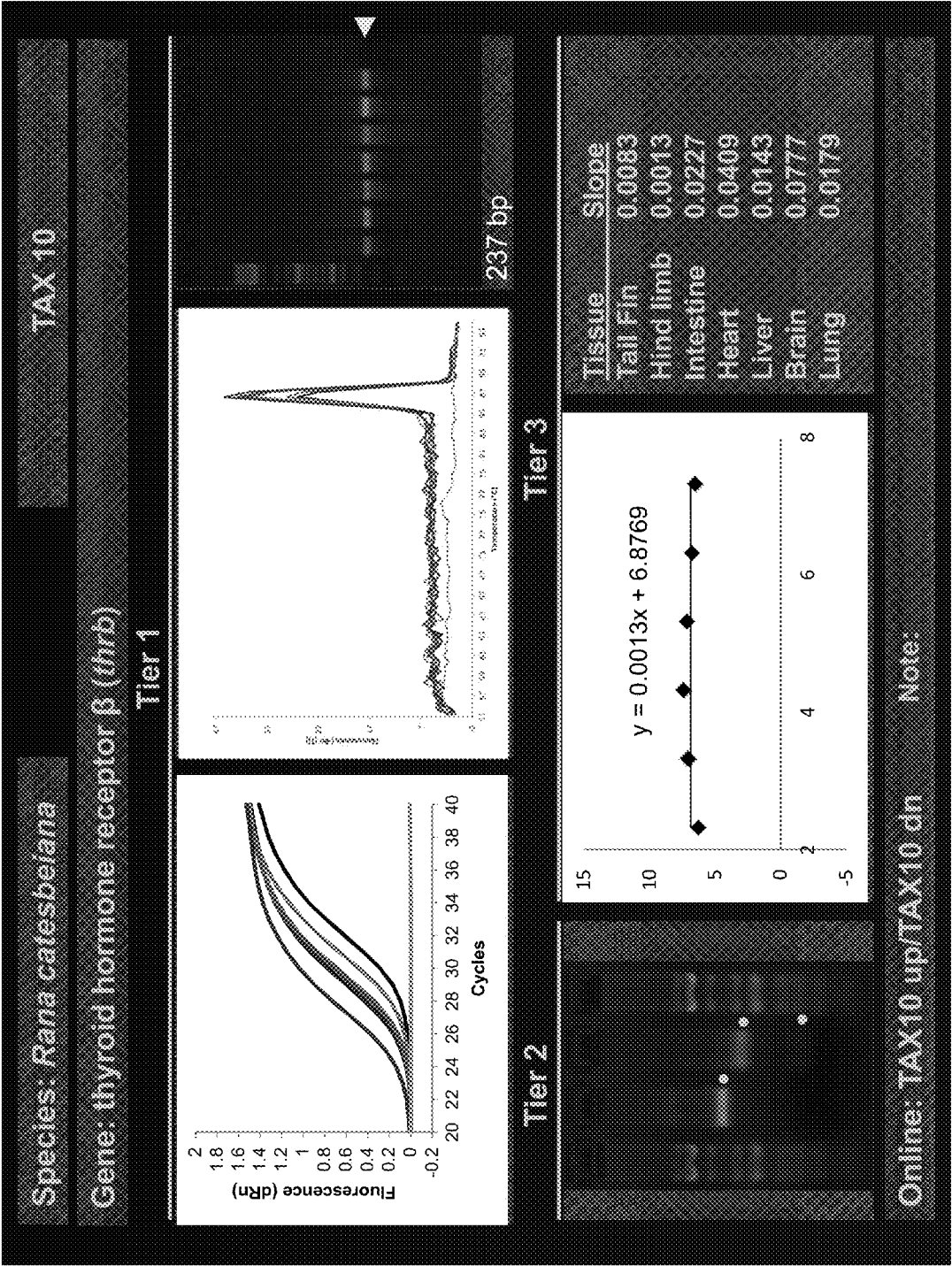


FIG. 2B(1)

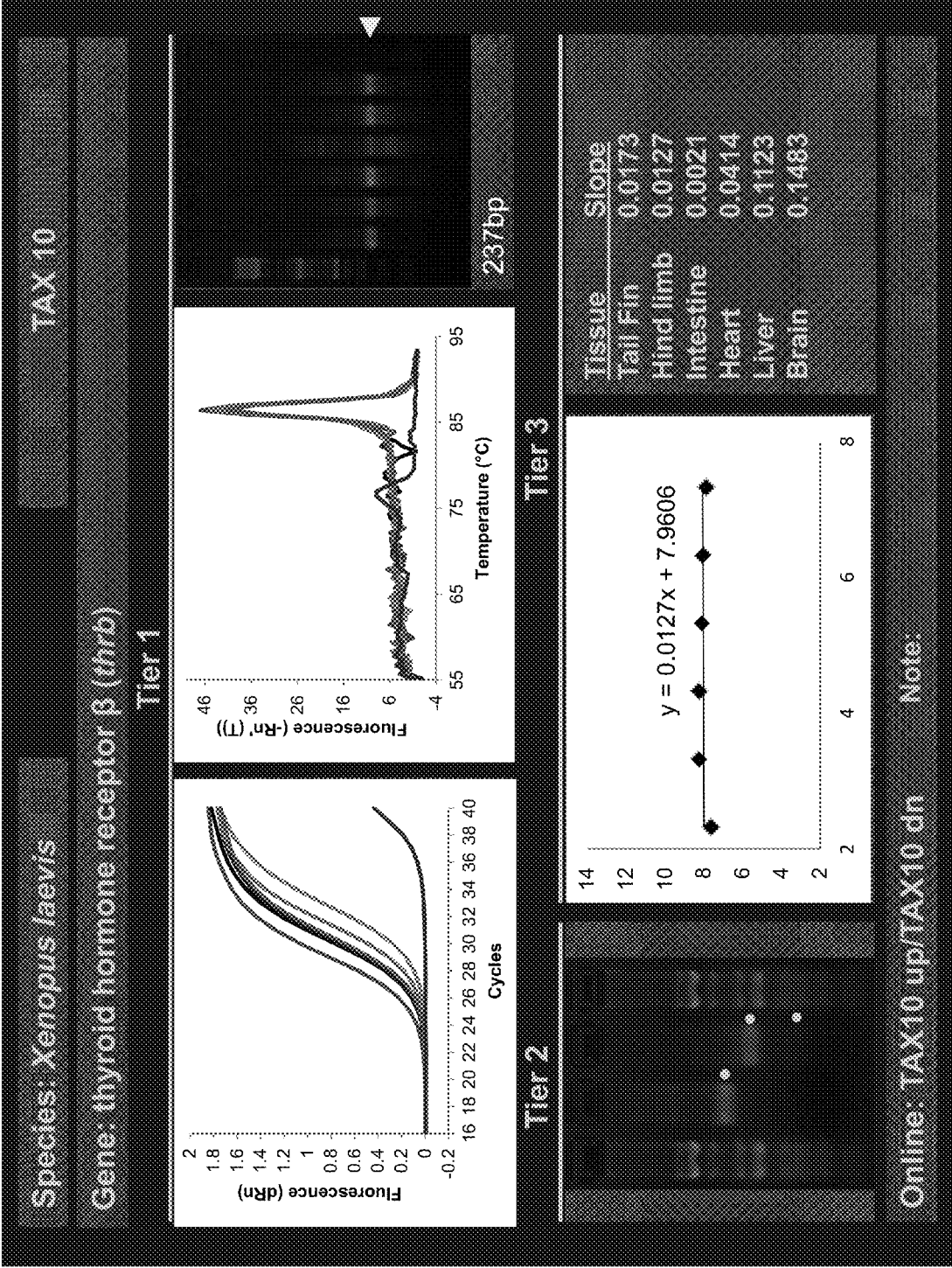


FIG. 2B(2)

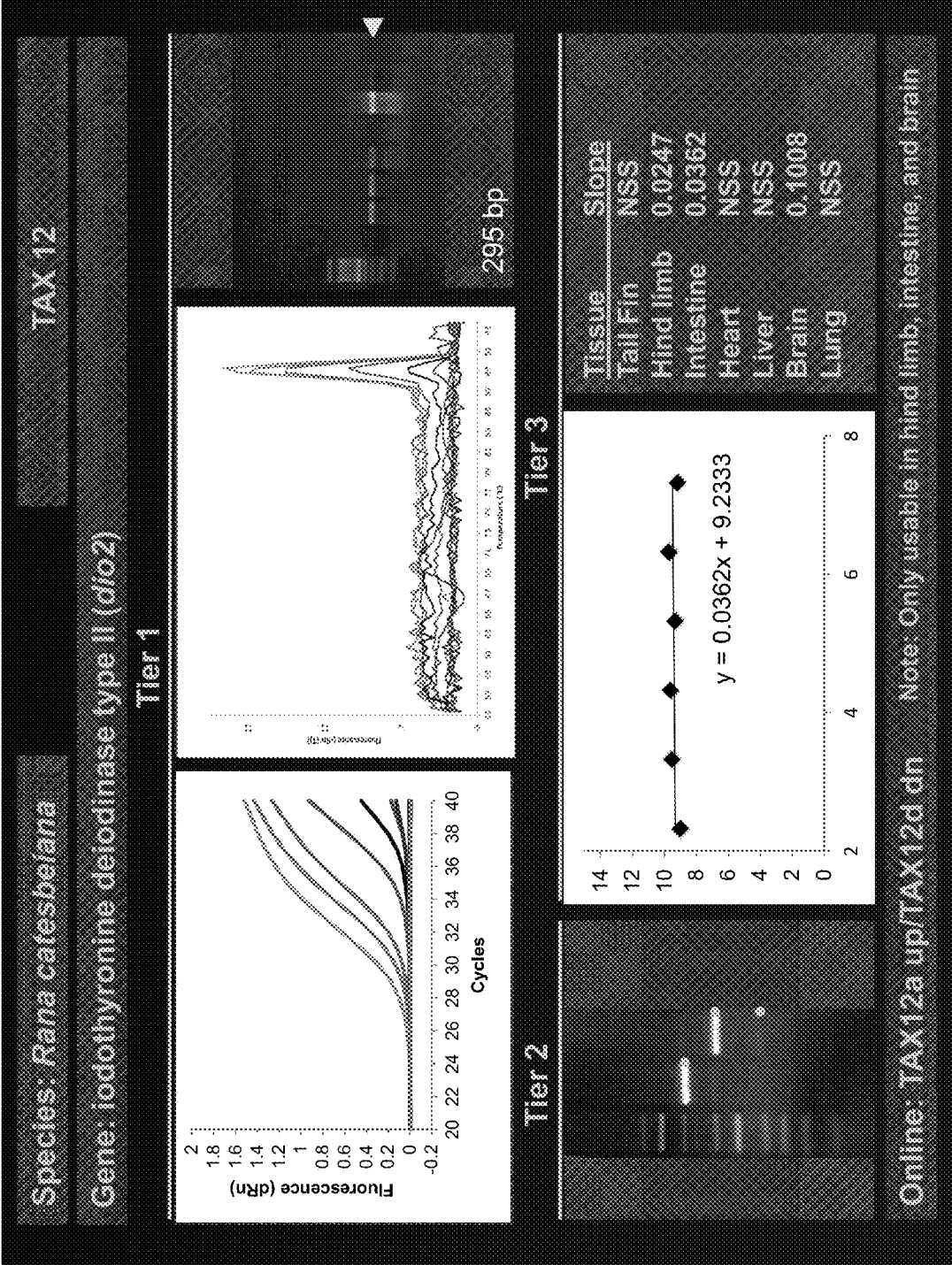


FIG. 2B(3)

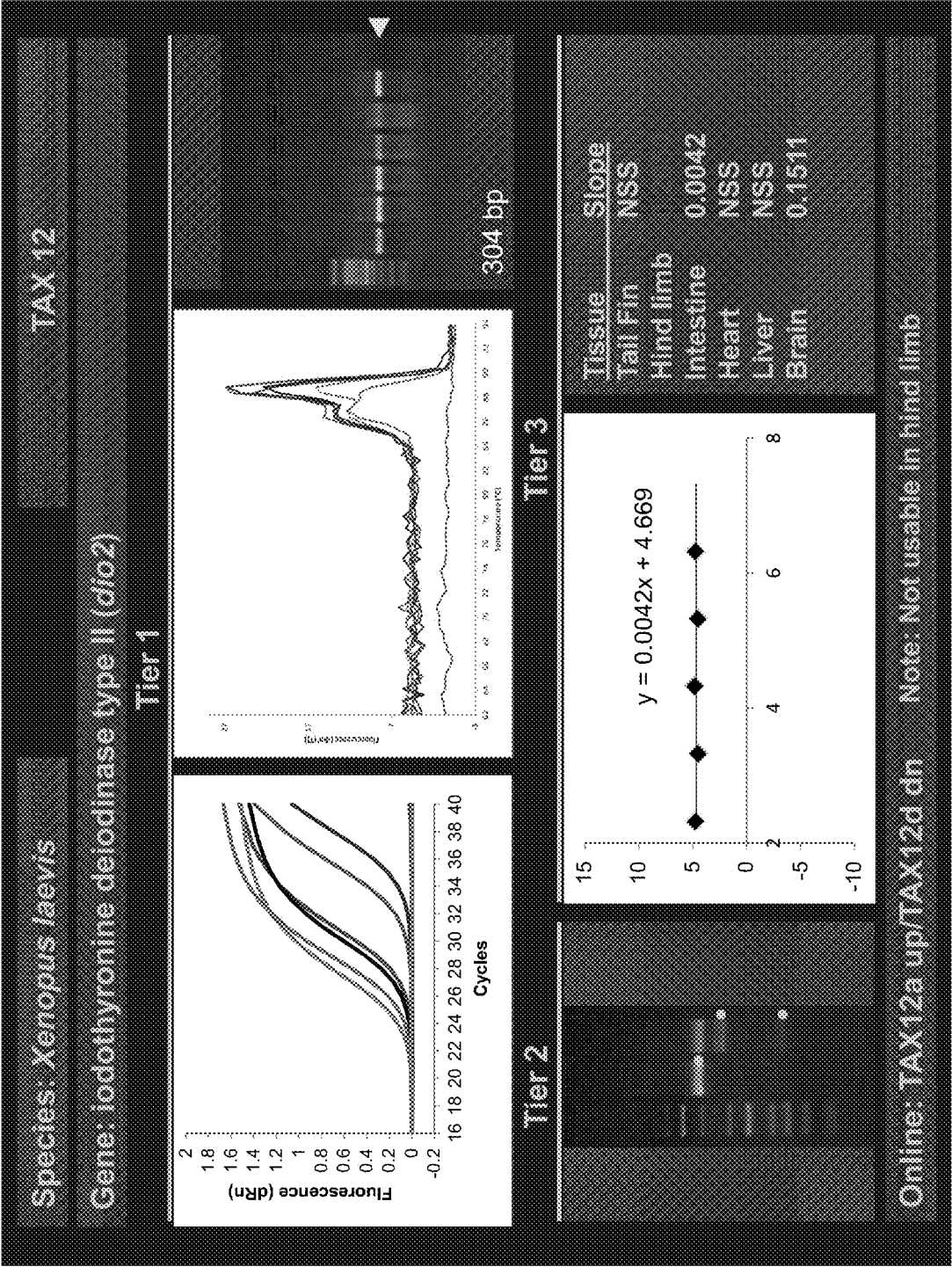


FIG. 2B(4)

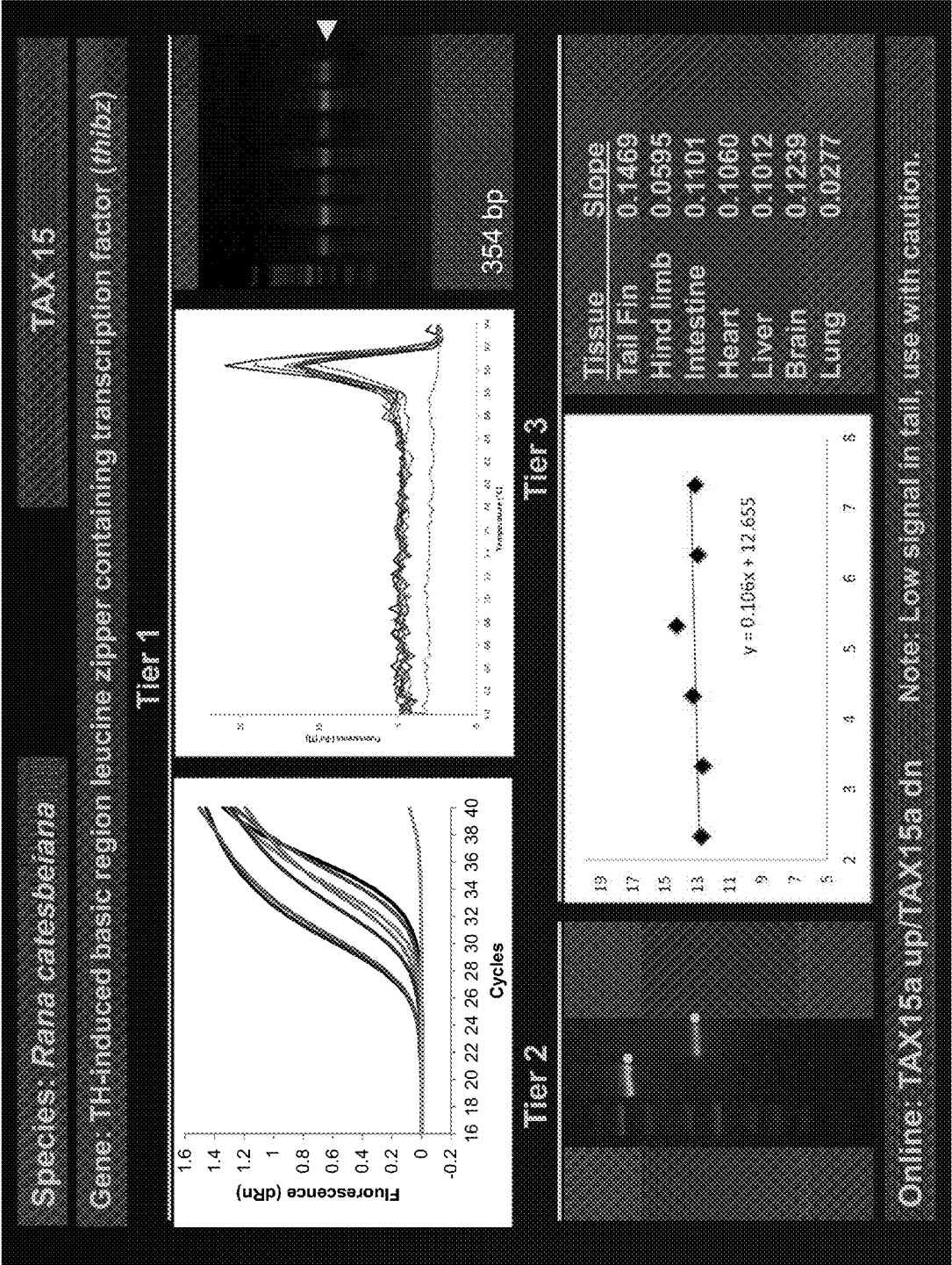
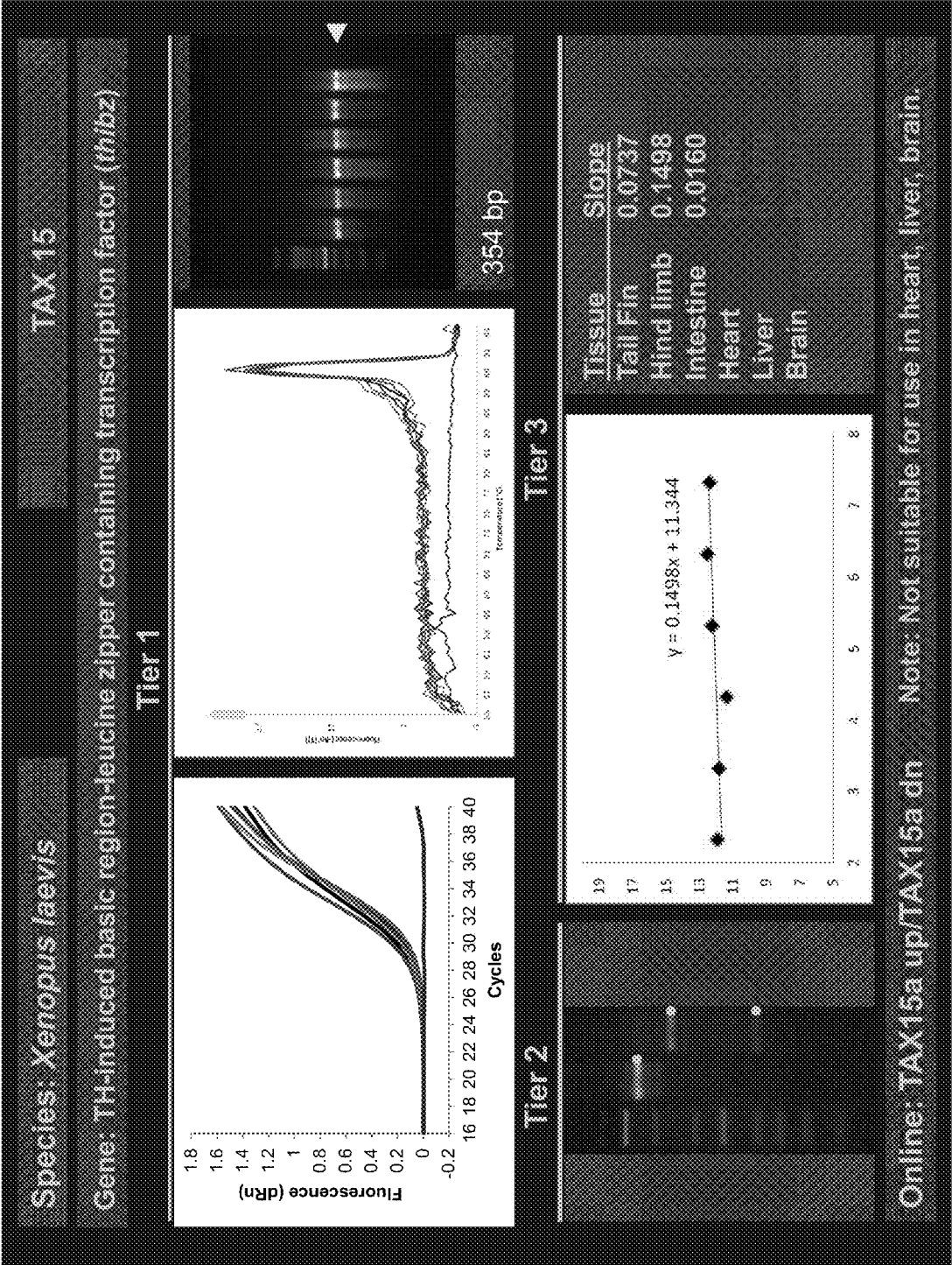


FIG. 2B(5)



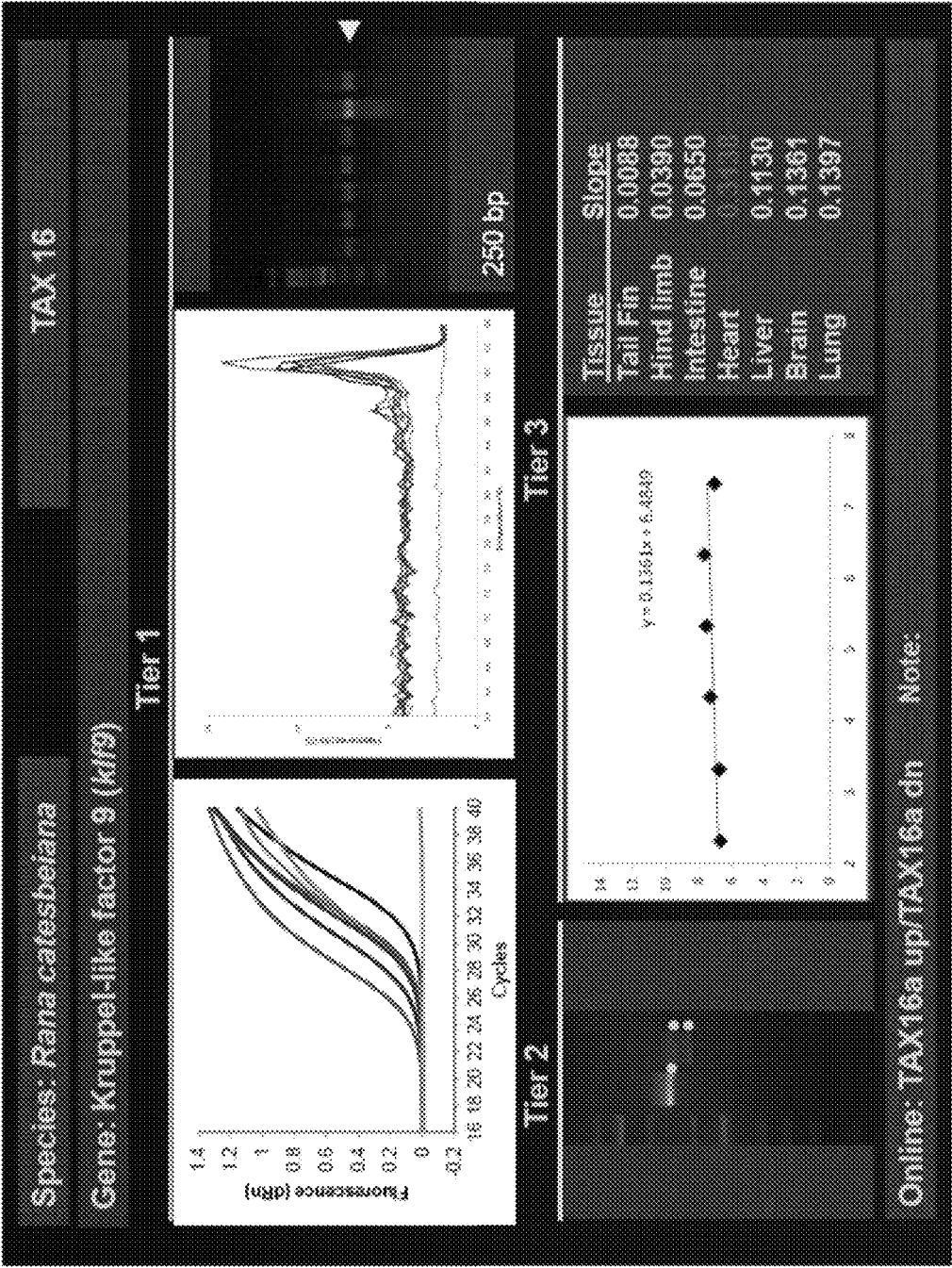


FIG. 2B(7)

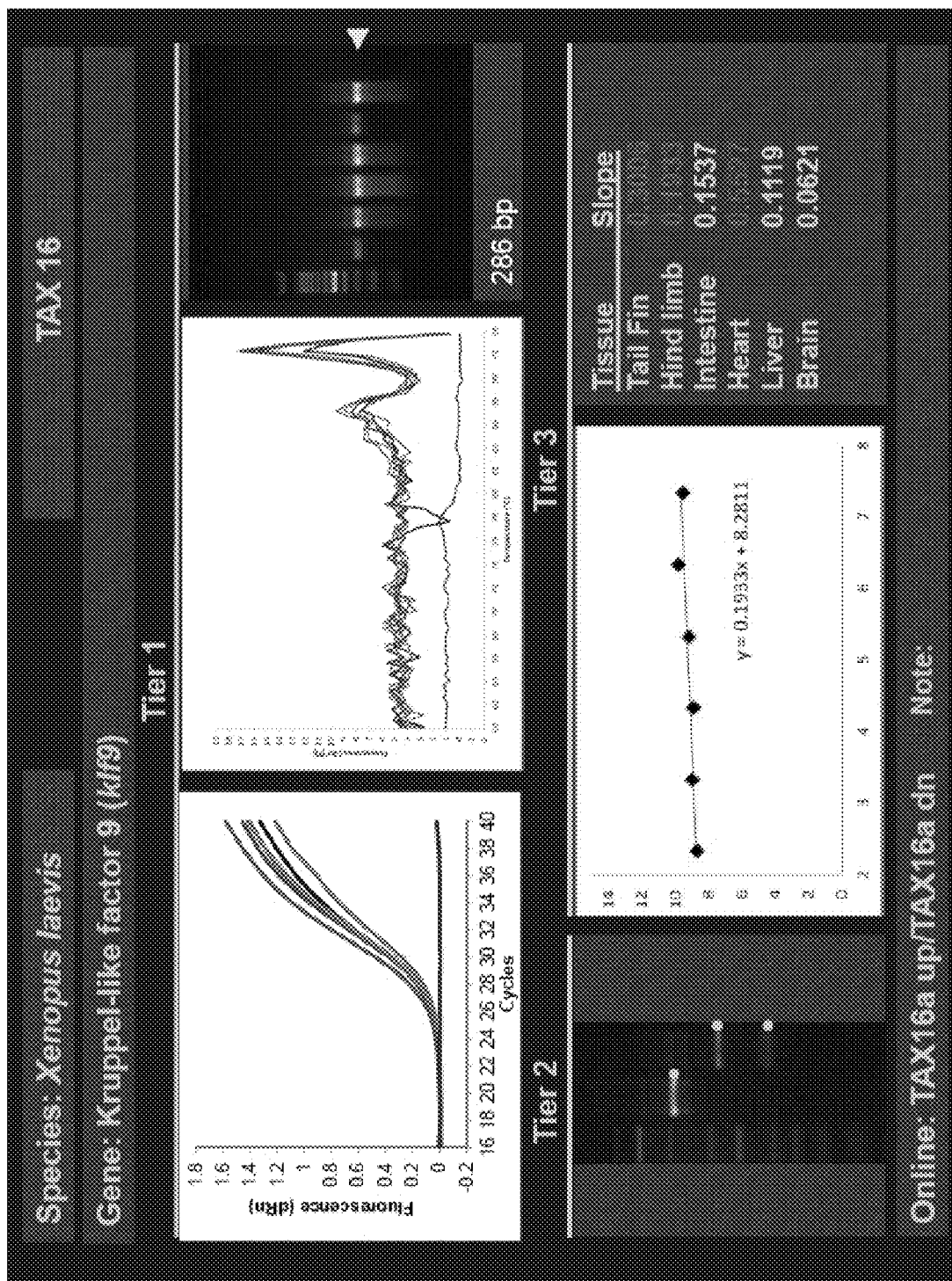


FIG. 2B(8)

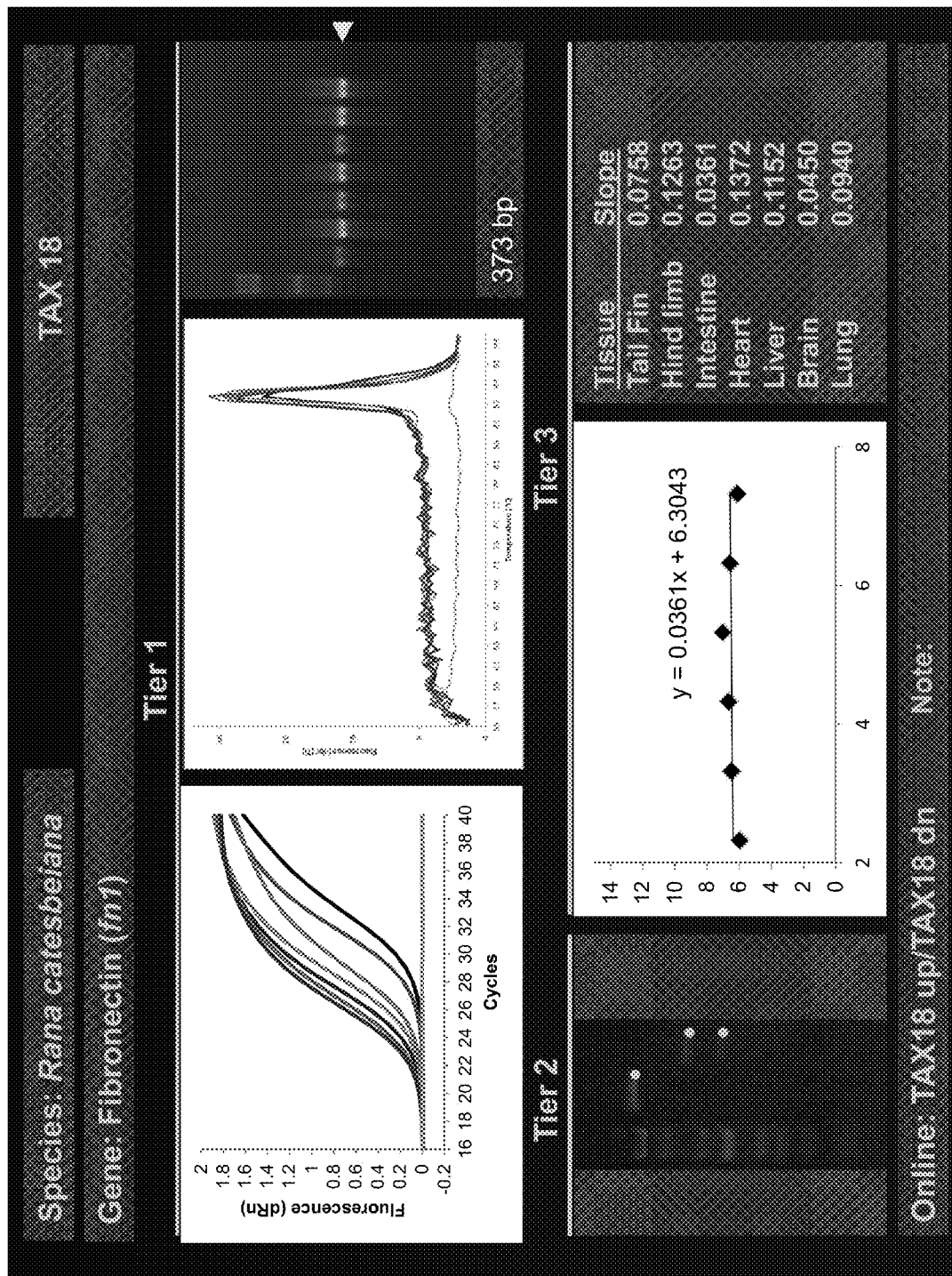
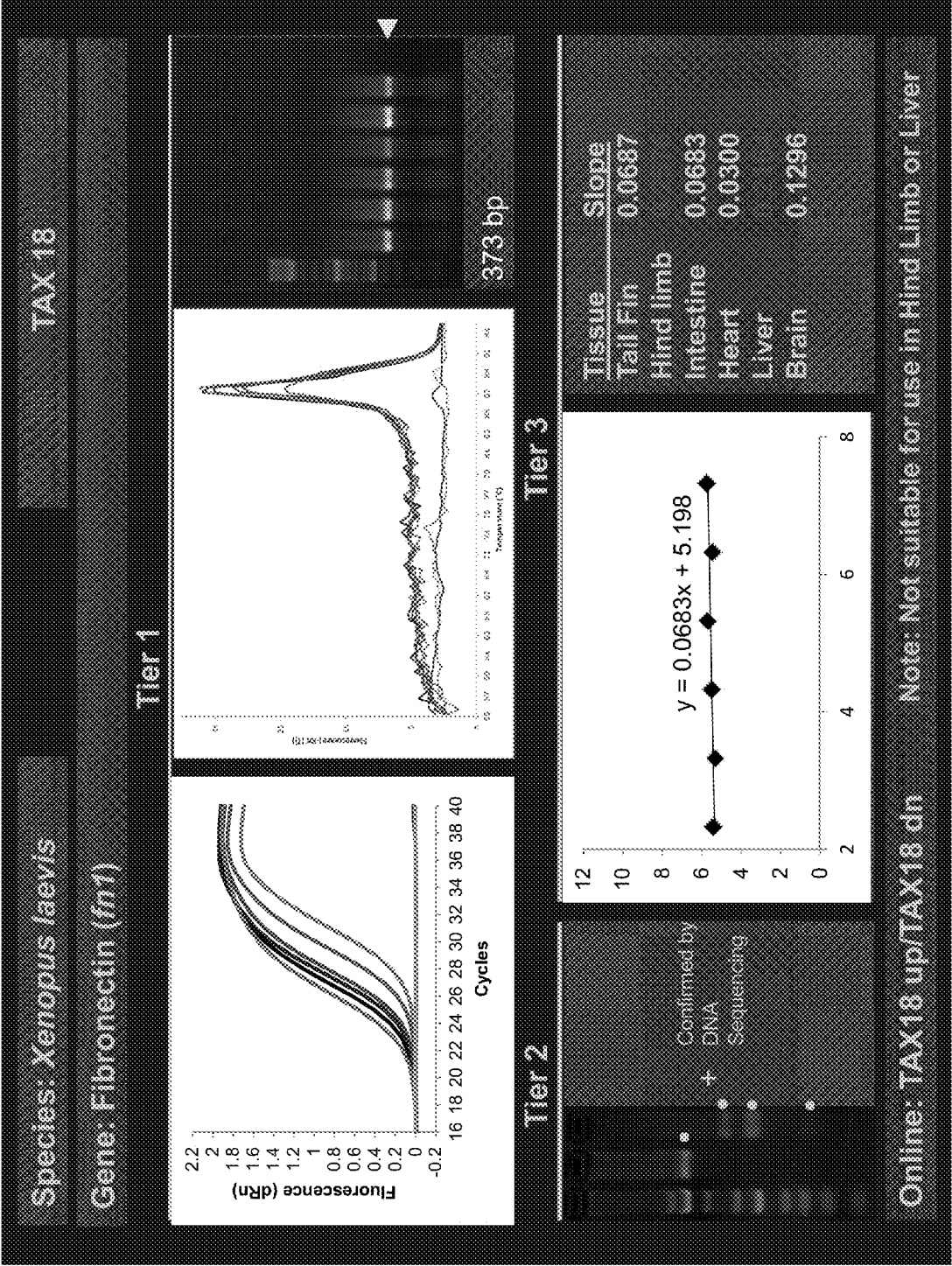
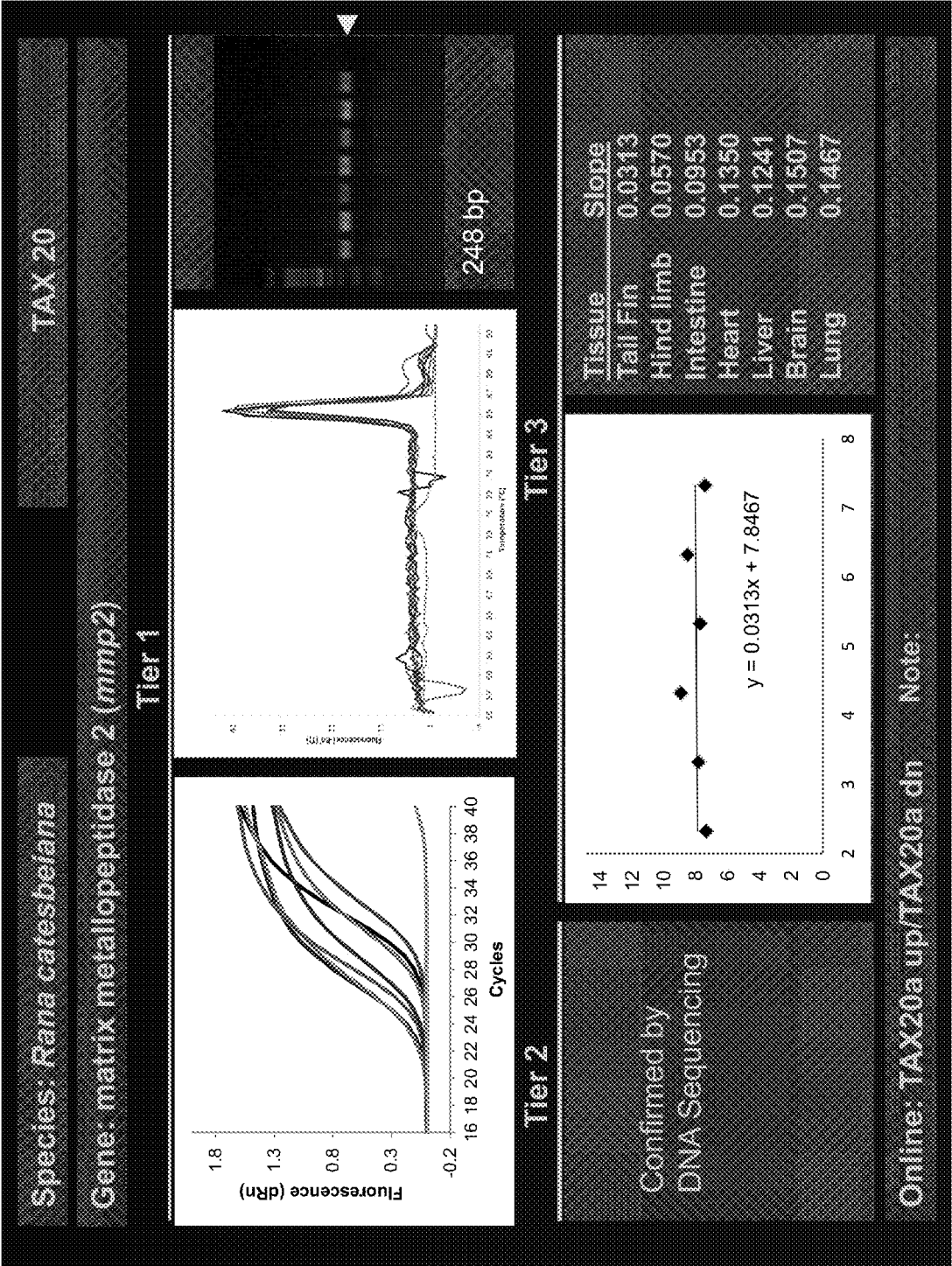


FIG. 2B(9)





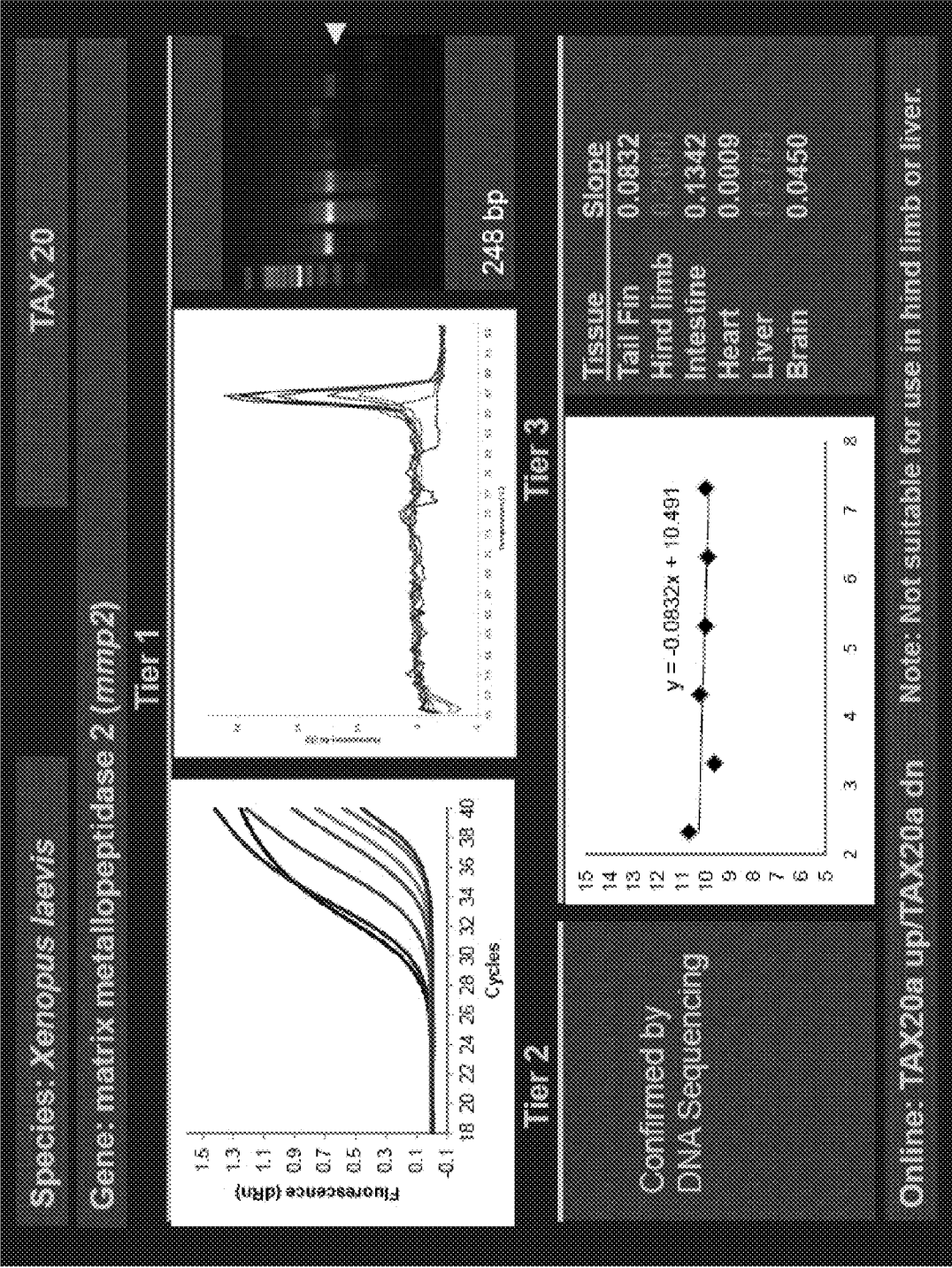
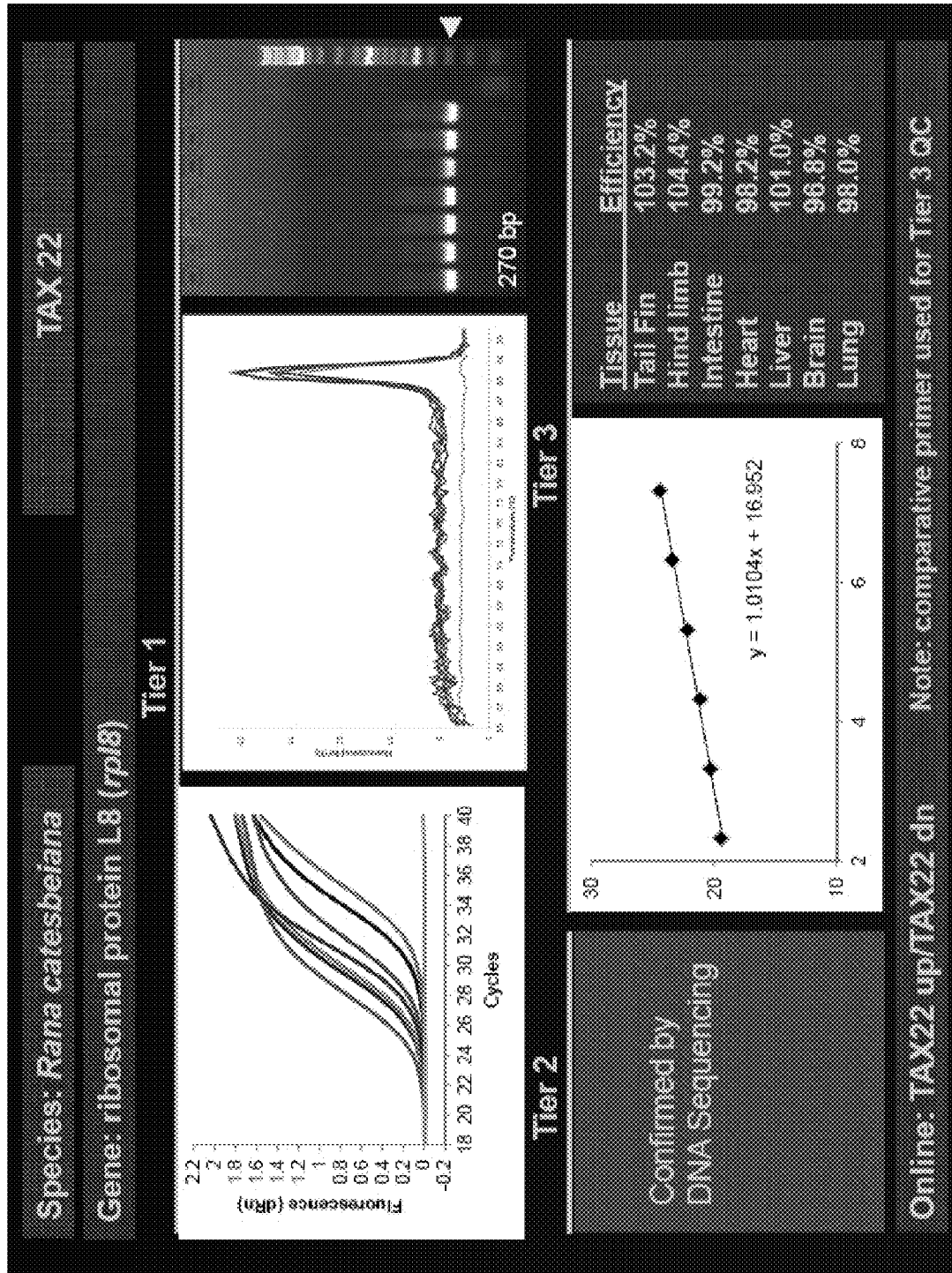
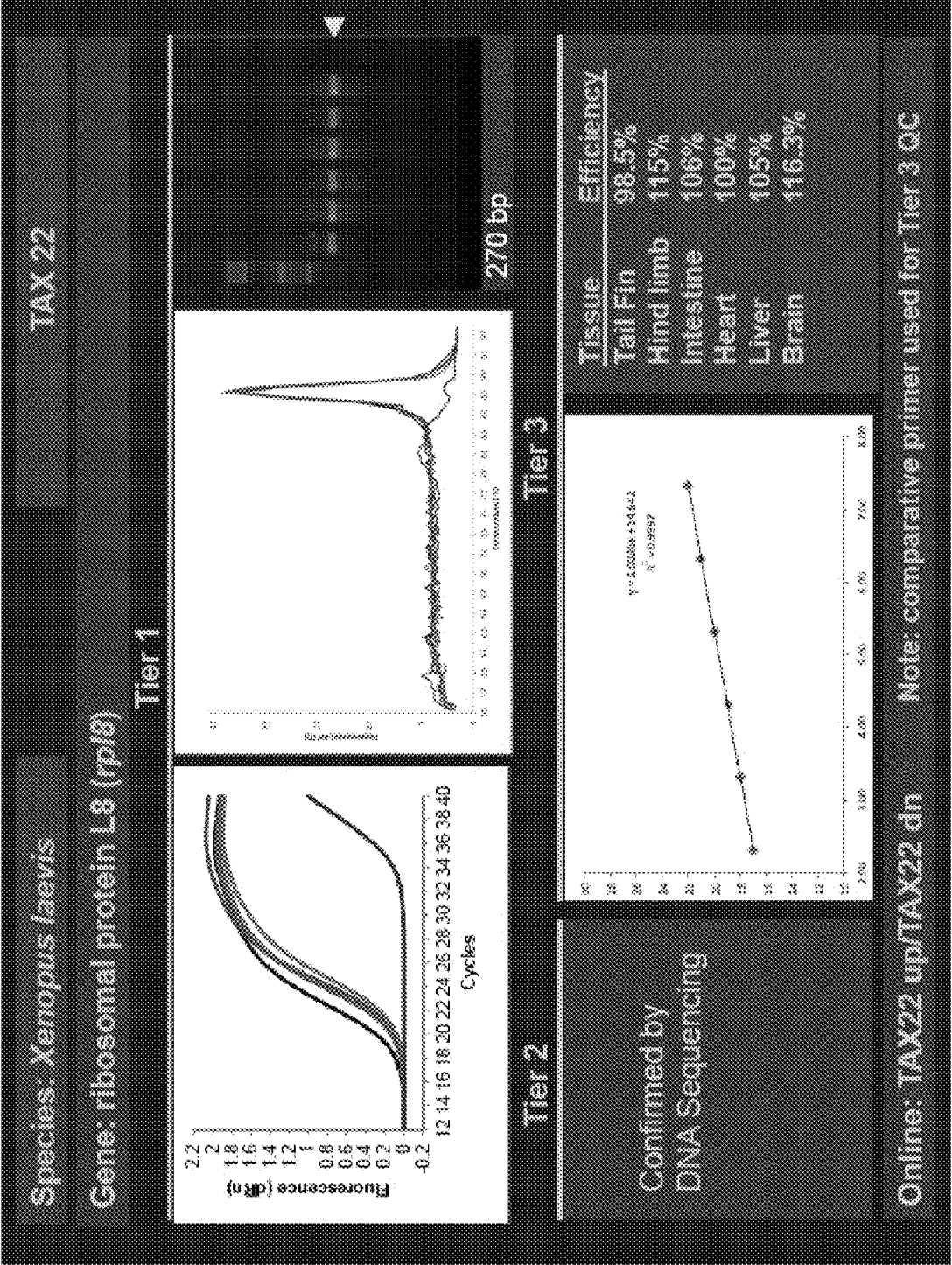
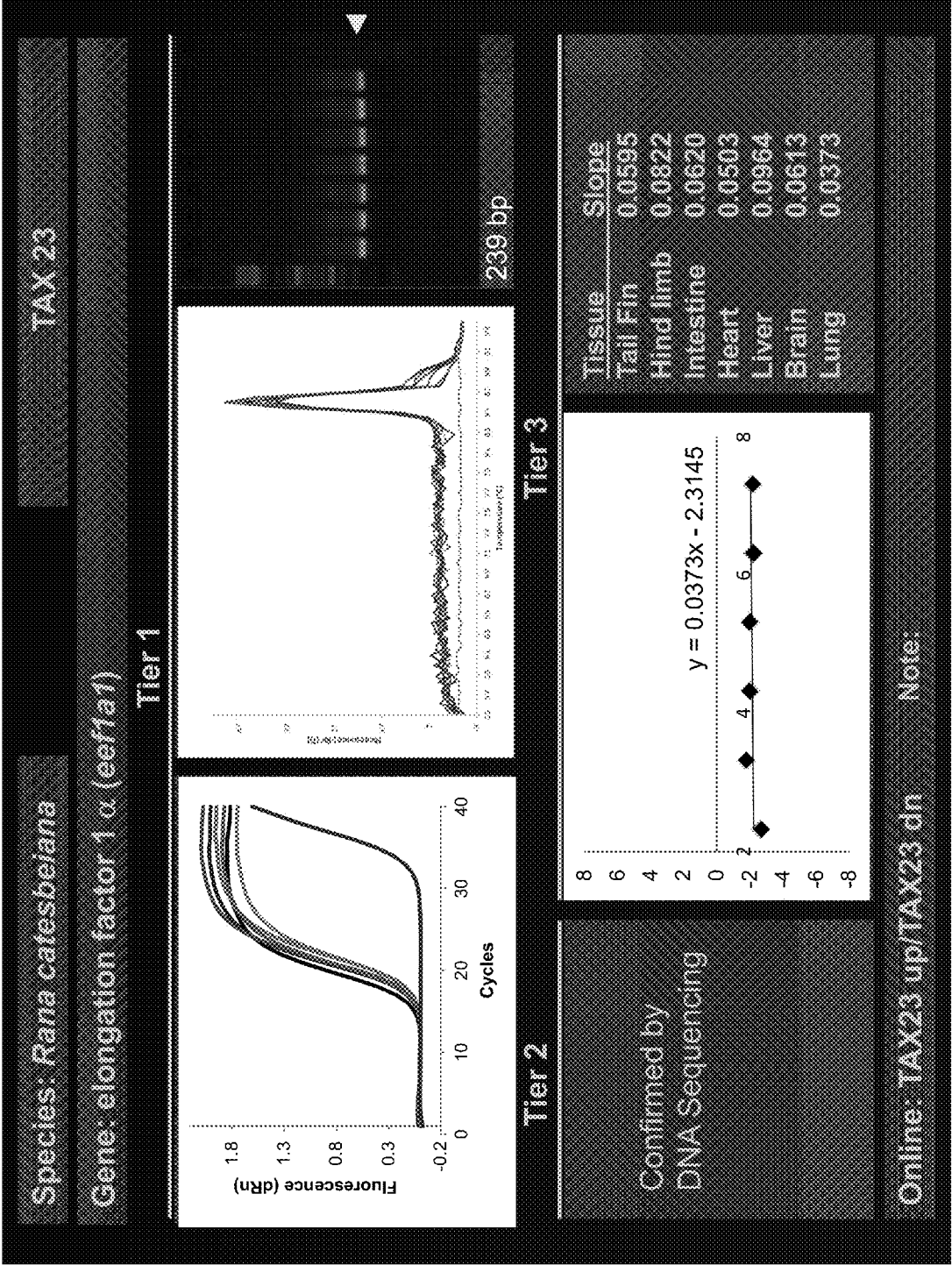


FIG. 2C(2)







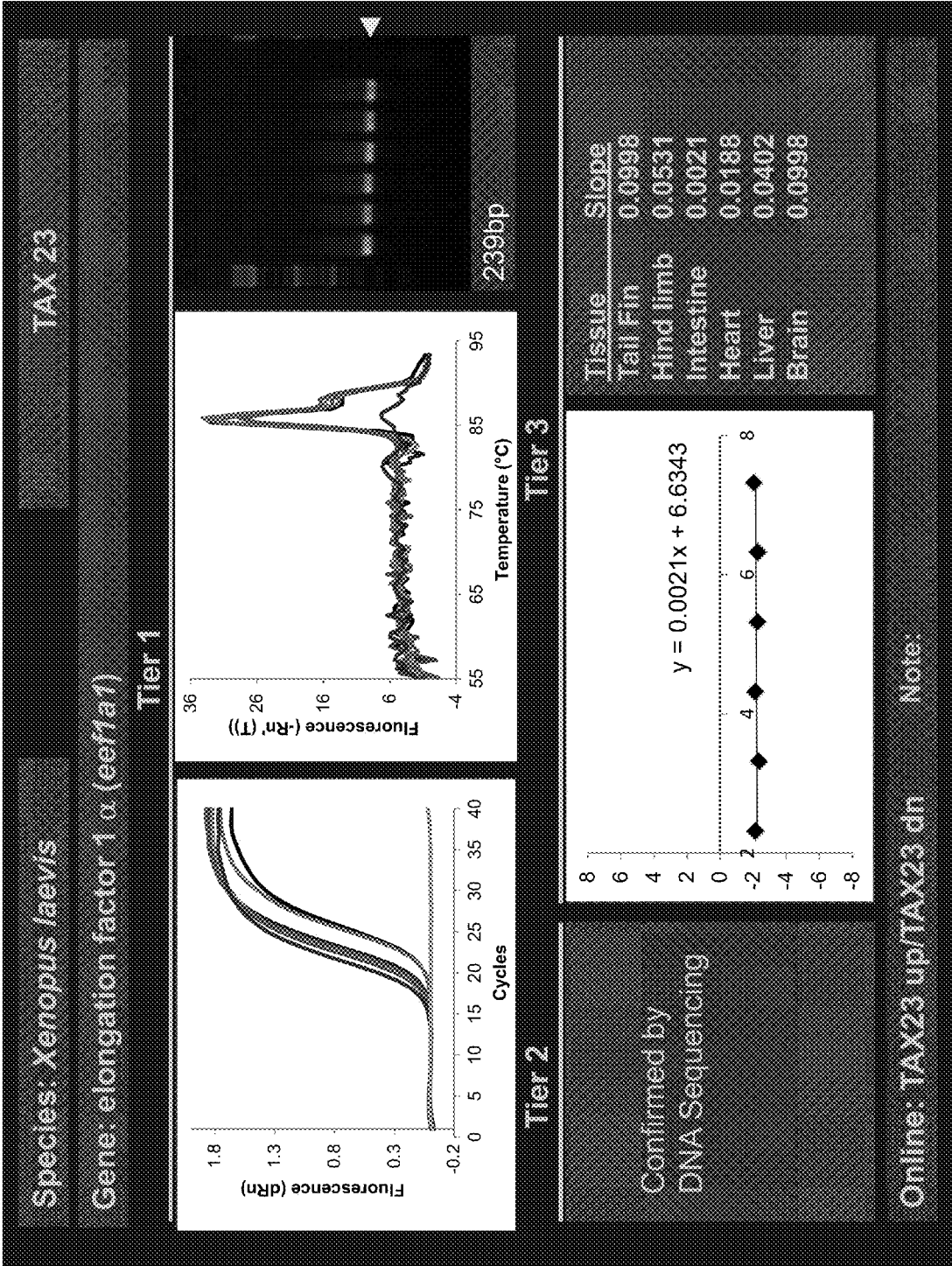
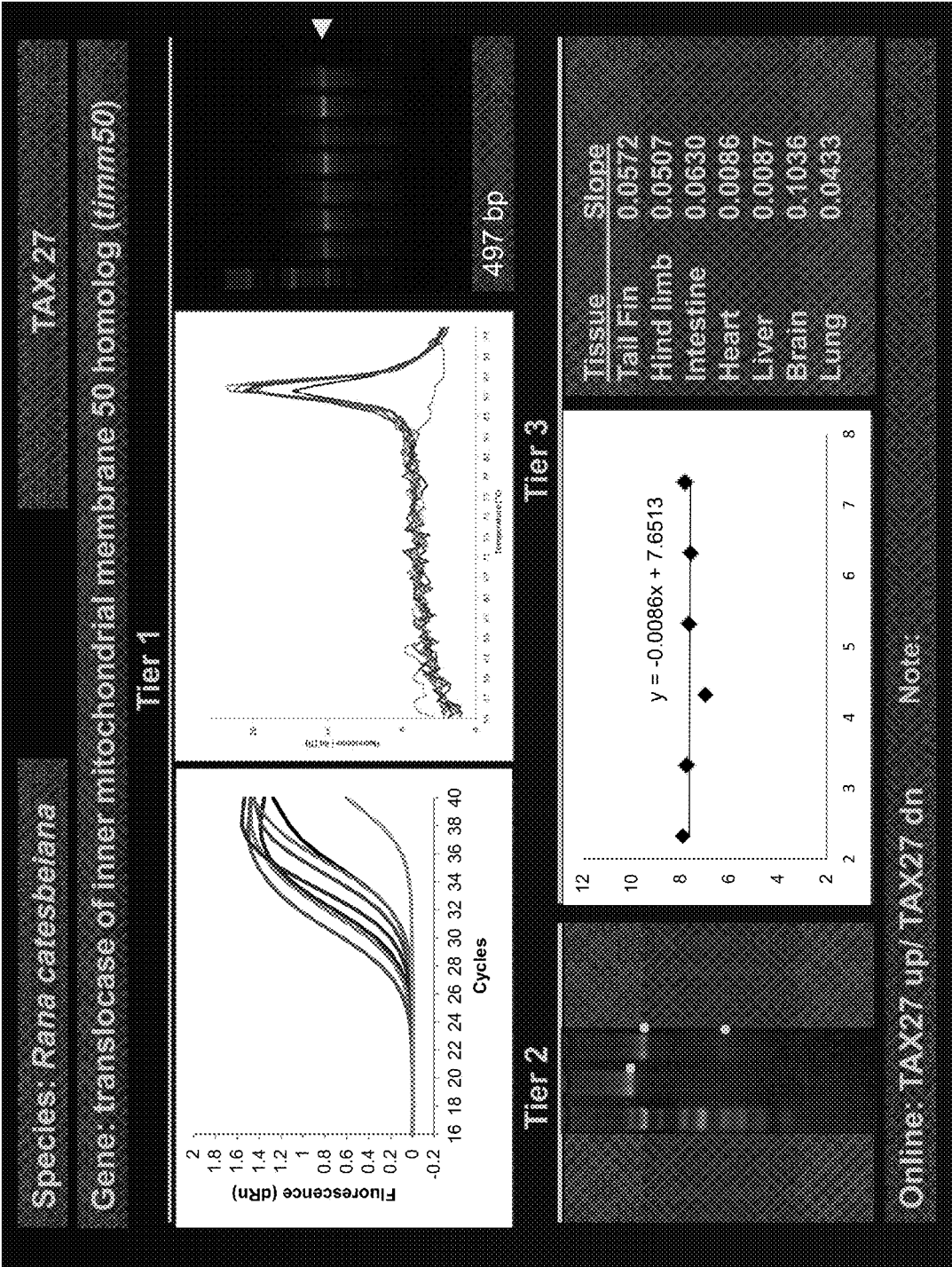
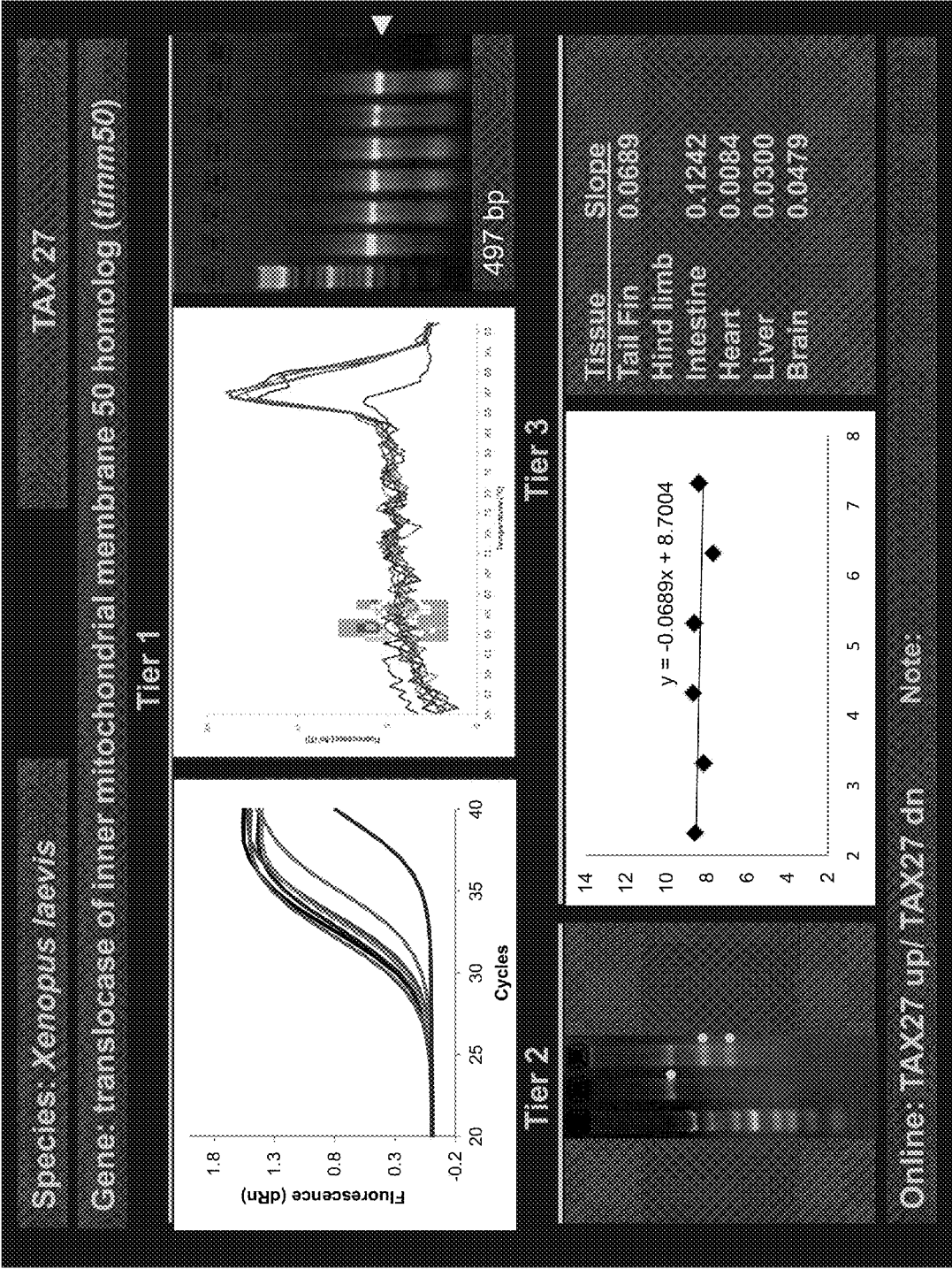


FIG. 2C(6)





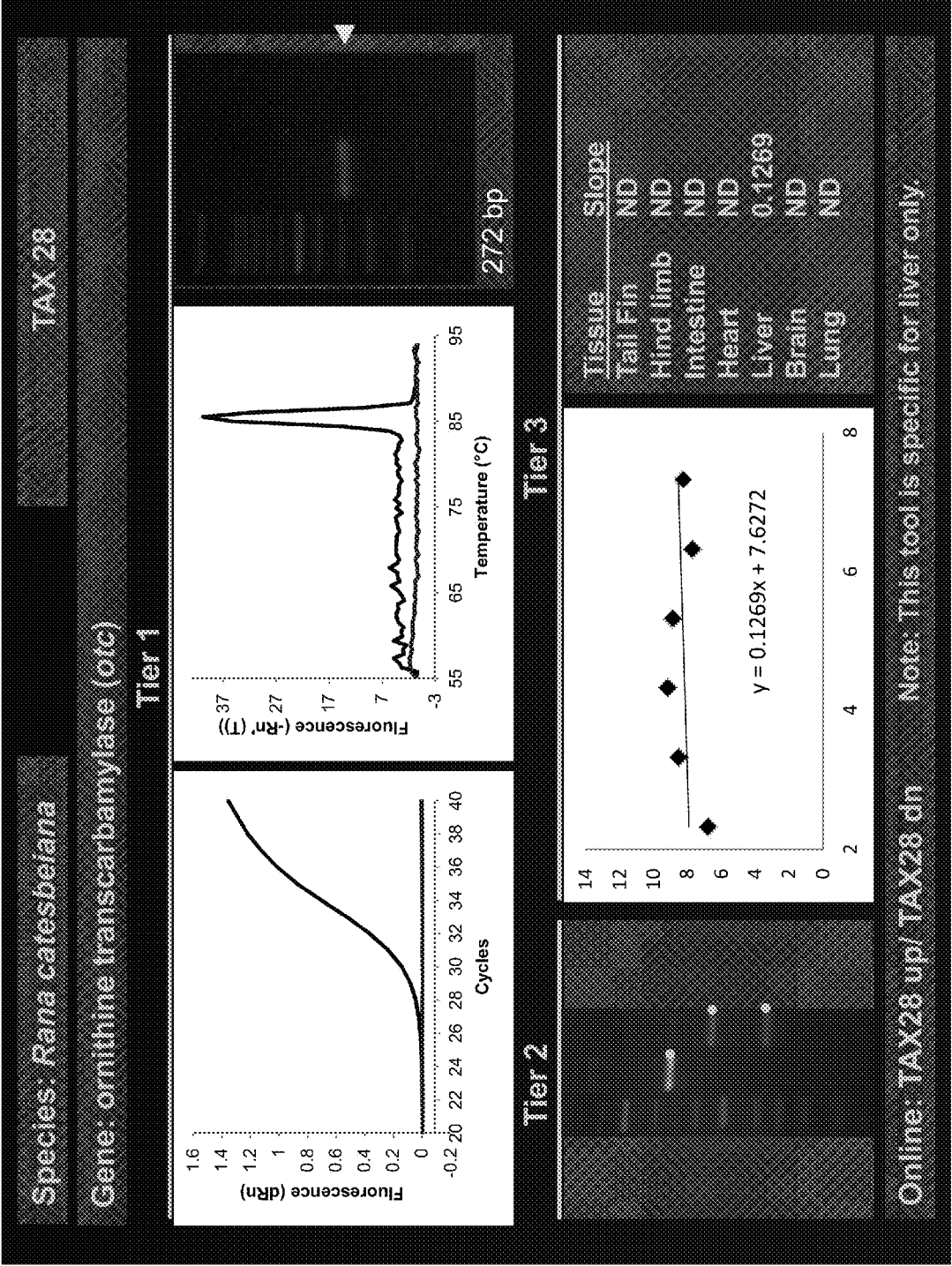


FIG. 2C(9)

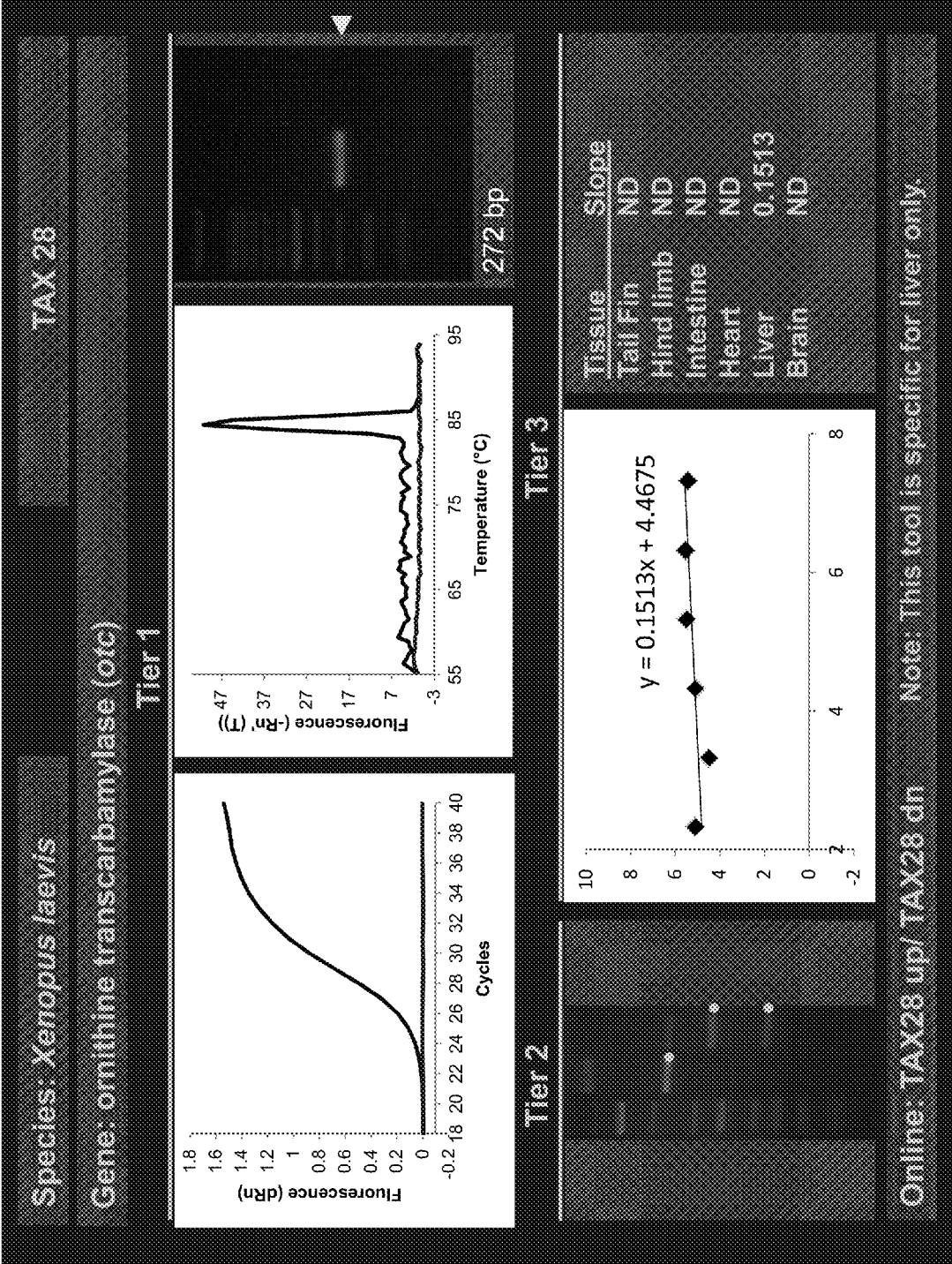


FIG. 2C(10)

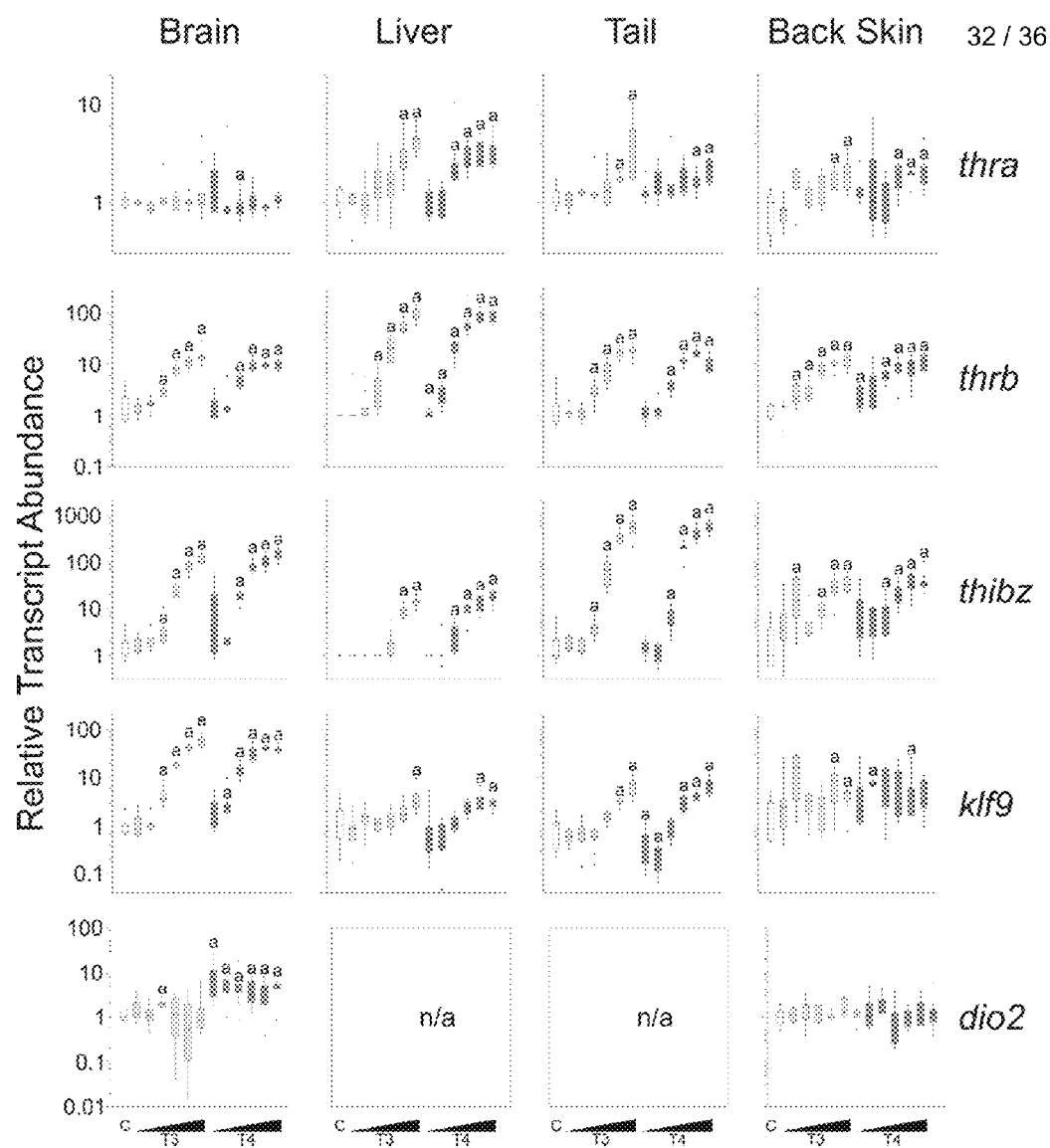


FIG. 3

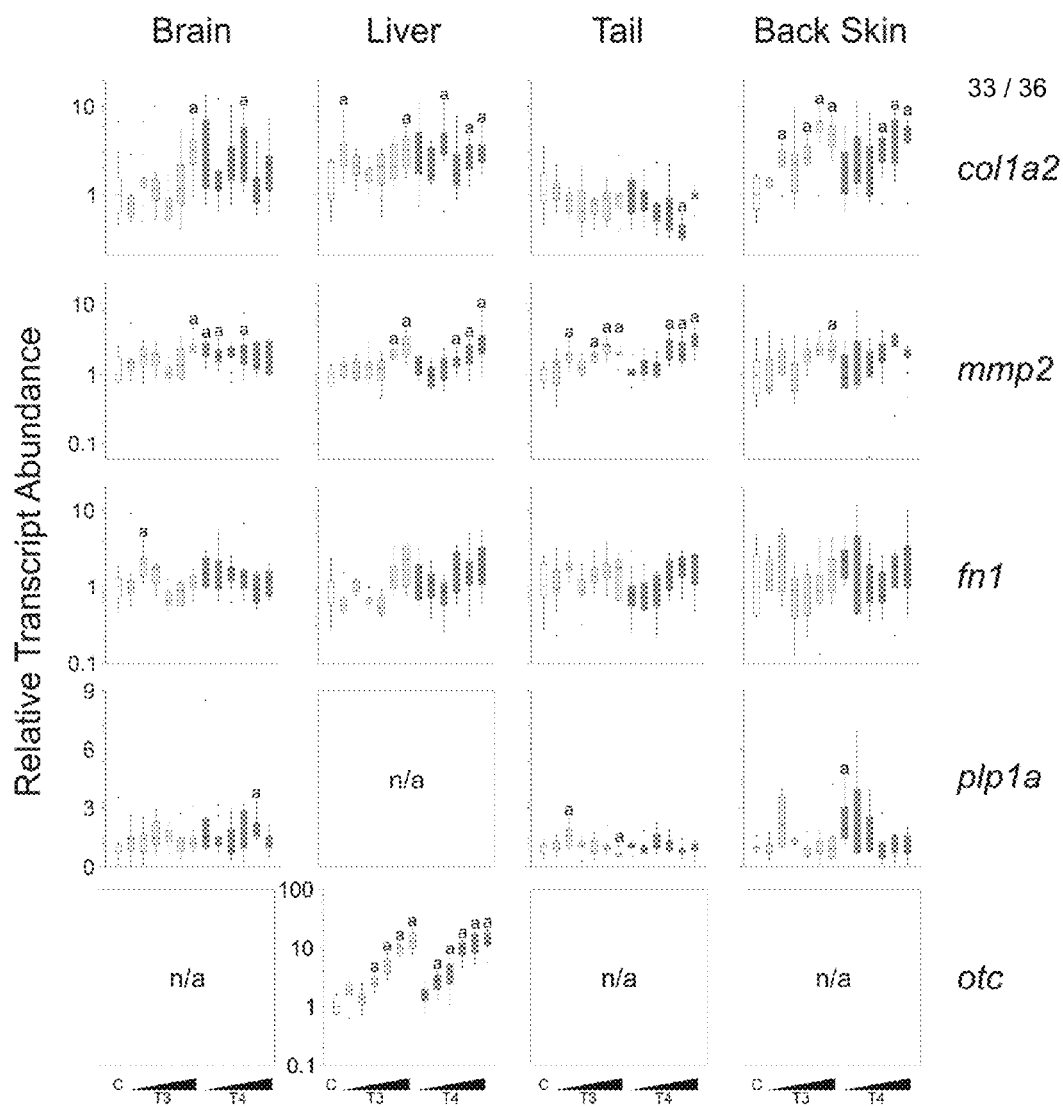


FIG. 4

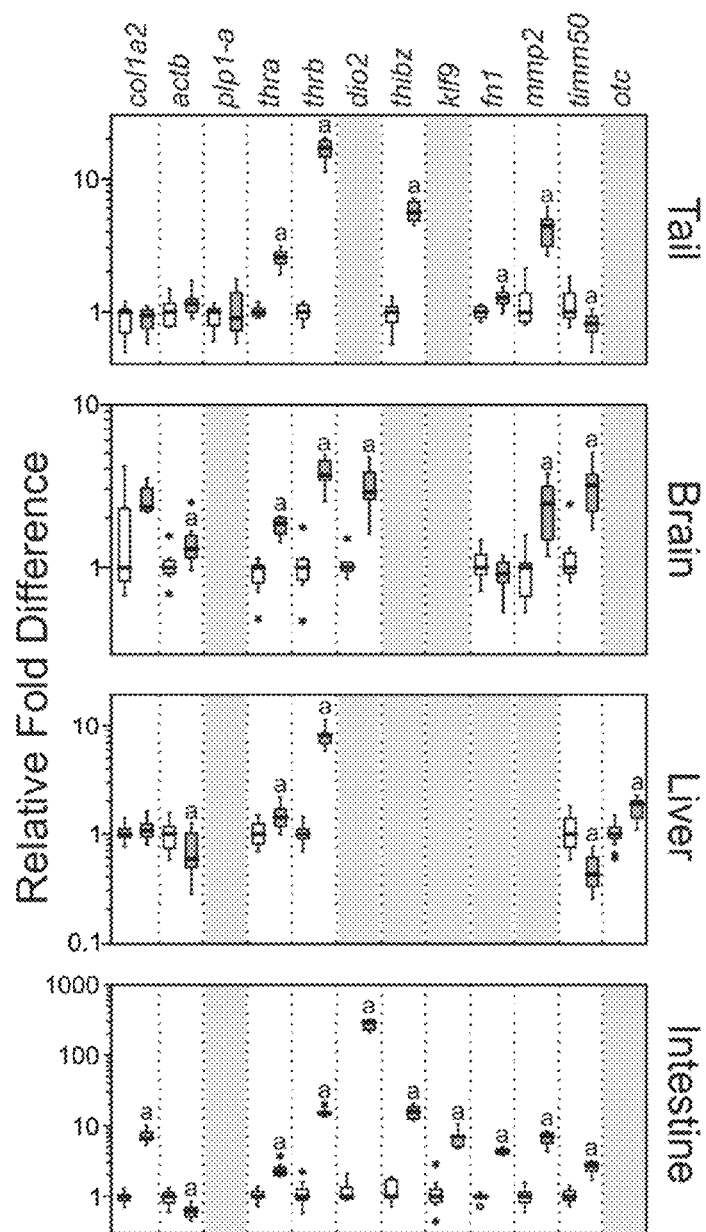


FIG. 5

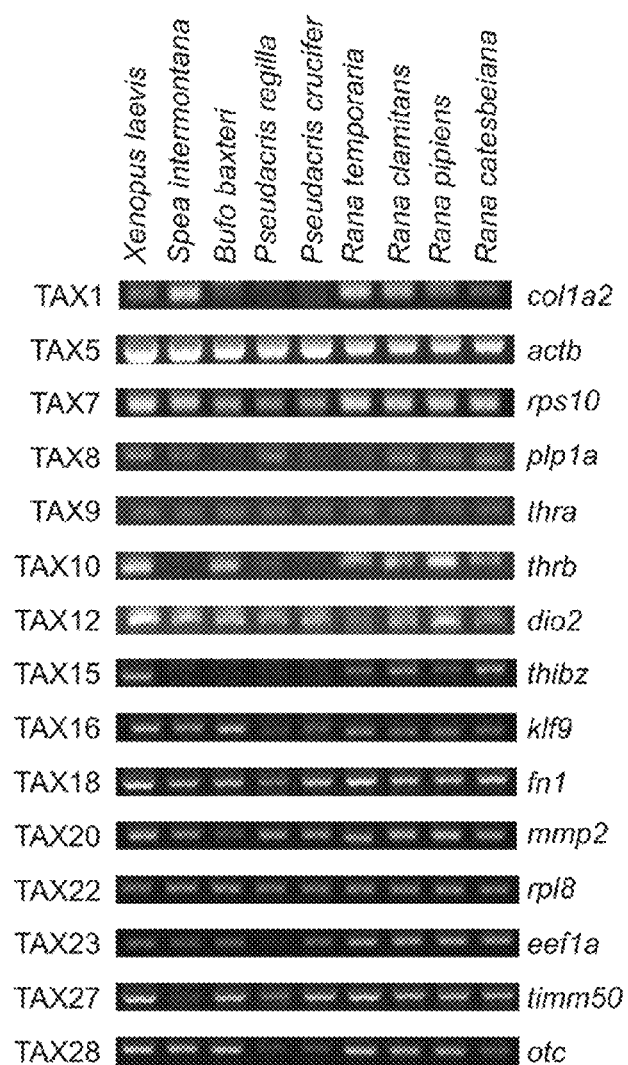


FIG. 6

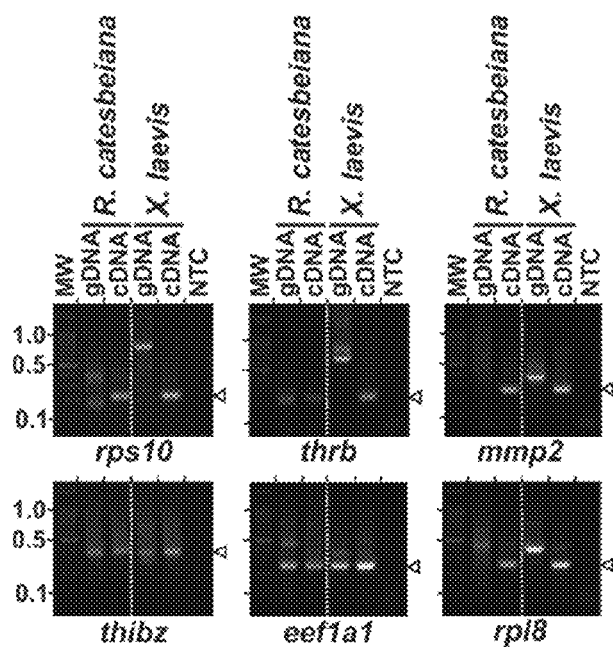


FIG. 7

Gene Targets		<i>Rana catesbeiana</i>							<i>Xenopus laevis</i>						Tissue Restrictions
		T	HL	I	H	LI	B	Lu	T	HL	I	H	LI	B	
TAX1	col1a2	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Clean Signal ~ Use Freely
TAX5	actb	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX7	rps10	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Non-specific Signal ~ Do Not Use
TAX8	plp1	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX9	thra	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Restricted Detection Range ~ Use With Caution
TAX10	thrb	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX12	dlx2	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Clean Signal ~ Use Freely
TAX15	thlb2	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX16	klf9	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Non-specific Signal ~ Do Not Use
TAX18	ln1	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX20	mmp2	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Restricted Detection Range ~ Use With Caution
TAX22	rpl8	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX23	sef1a1	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Clean Signal ~ Use Freely
TAX27	tlmm50	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	
TAX28	otc	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	Non-specific Signal ~ Do Not Use
		⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	⊙	

FIG. 8

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## ANURAN CROSS-SPECIES MOLECULAR SENSORS

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 61/883,138, filed Sep. 26, 2013, the entire disclosure of which is hereby incorporated by reference.

### FIELD OF THE INVENTION

The invention relates to biological assays and the study of animal development, environmental toxicology and ecological assessments.

### SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Dec. 12, 2014, is named 026814.0034\_SL.txt and is 7,129 bytes in size.

### SUMMARY OF THE INVENTION

The invention describes the DNA primer sequences designed for the determination of gene or transcript information from Anuran species. There are thousands of Anuran species known worldwide and obtaining genetic information from each species for the development of quantitative real time polymerase chain reaction (qPCR) assays is not feasible. Our invention is comprised of one or more specially-designed primer pairs that can be applied to diverse Anuran species spanning over hundreds of million years in evolution, including those species commonly used in laboratory studies for development and/or toxicity testing and sentinel species used for environmental toxicology or ecological assessment. Described herein are optimized conditions for use of these primers in examples of Anuran tissues. Another aspect of the invention is a rapid, sensitive, high-throughput assay useful for supporting potential risk assessment across vertebrate clades, and that is also useful for evaluation of complex contaminant mixtures.

### BACKGROUND

Amphibians are used extensively as scientific model organisms, in health and environmental research, as test organisms, and have an undeniable role as sentinel species, as a food source, and in insect control. In contrast to mammals with extensive genomic resources, a particular challenge exists for evaluating gene expression endpoints in amphibian species where clades exhibit evolutionary divergence of over 300 million years (AmphibiaWeb 2012). Of the amphibians, the Anura-representing frogs and toads are the most numerous on the planet representing over 6,000 of the ~7,000 known species (AmphibiaWeb 2012). Despite this impressive number, only two closely-related Pipid species, *Xenopus laevis* and *Xenopus tropicalis*, have sufficient genomic resources for gene expression studies. Yet many other species including ones that have diverged over 200+ million years ago, serve as important species, regionally and globally. There is a lack of the most minimal genomic information leading to a significant investment in time and resources to even clone a portion of a single gene in order to develop validated gene expression tools for a species of interest. To circumvent this difficulty, we have developed a suite of qPCR-ready primer sets that identify particular genes and/or their transcripts. Each primer pair has been

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validated to function under stringent criteria in species as diverse as Pipids and Ranids. The use of these primer sets provides a simple, low-cost solution to the issue of cross-species comparison of responses and sensitivities.

Endocrine disruptors (EDCs) are chemicals, either environmental or man-made, that disturb the endocrine signaling pathways of humans and wildlife. EDC exposure often results in cancer, fertility problems, and other diseases (Vandenberg, Colborn et al. 2012). Therefore, the risk from these exposure effects has led to a great need for sensitive and appropriate methods for indicating deleterious EDC effects. For this reason, there is considerable interest in developing novel diagnostic assays to detect EDCs in the environment as well as in products meant for human consumption or that are used to package food products such as plastic food containers. Over 80,000 chemicals are registered for use in the US (NIEHS 2013) and there is increasing concern regarding their impact since it is now established that EDCs do not follow the classical rule of “the dose makes the poison” (Vandenberg, Colborn et al. 2012). Rather non-monotonic responses and low-dose effects are actually common in studies of natural hormones and EDCs and clear linkages between environmental exposures to EDCs and human diseases/disabilities are becoming evident (Vandenberg, Colborn et al. 2012). EDCs are generally found in low concentrations in the environment, but even minute quantities can have demonstrable impact as hormone disruptors. In fact biological activity can be detected below current analytical detection limits and measurement of EDCs in the context of complex mixtures such as municipal wastewater effluent is not necessarily a good predictor of biological activity (Quanrud and Propper 2010). Given that many aquatic ecosystems contain significant concentrations of environmental contaminants (Kolpin, Furlong et al. 2002), and that many of these compounds and/or their metabolites have been detected in human plasma (National Report on Human Exposure to Environmental Chemicals, Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, <http://www.cdc.gov/exposurereport>), we have developed a suite of tools for indicating deleterious EDC effects. Such screens can be used as a first level evaluation of exposure health risk for both wildlife species and humans. Most of what we know about EDCs pertains to substances that disrupt estrogen signaling pathways and technologies to detect estrogenic EDCs have been developed (Van Aggelen, Ankley et al. 2010; Hecker and Hollert 2011).

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a cladogram depicting certain major families of the order Anura.

FIGS. 2A(1) through 2A(10); 2B(1) through 2B(10) and 2C(1)-2C(10) illustrate the results of the three-tiered quality assurance/control procedure demonstrating activity of the primer sets according to an embodiment of the invention.

FIG. 3 illustrates the results of a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Rana catesbeiana* according to an embodiment of the invention.

FIG. 4 is another illustration of the results of a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Rana catesbeiana* according to an embodiment of the invention.

FIG. 5 is an illustration of the results of a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Xenopus laevis* according to an embodiment of the invention.

FIG. 6 illustrates results of tests demonstrating that primer sets according to an embodiment of the invention are usable in a wide range of Anuran species.

FIG. 7 are illustrations of representative examples of primer set performance on genomic DNA across different Anuran species.

FIG. 8 is a summary of tissue types in which each primer set is usable in *Rana catesbeiana* and *Xenopus laevis*.

#### DETAILED DESCRIPTION OF THE INVENTION

The thyroid hormones (THs) are crucial for normal growth, development, and metabolism in all vertebrates including humans (Morreale de Escobar, Obregon et al. 2004; Zoeller and Rovett 2004). In fetal and neonatal mammals, environmental contaminants such as perchlorate and polychlorinated biphenyls (PCBs) impact TH action contributing to serious defects in development and organ function including cognitive and motor deficits, abnormal bone growth, cardiac problems, and altered lipid metabolism (Zoeller and Rovett 2004; Bernal 2005; Cheng 2005; Siesser, Cheng et al. 2005; Liu and Brent 2010). The brain is particularly vulnerable (Porterfield 1994; Brucker-Davis 1998; Jones, Thoemke et al. 2005; Zoeller 2005; Zoeller and Crofton 2005) and the impact of mild hypothyroidism during critical developmental phases can have significant societal costs (Miller, Crofton et al. 2009). In amphibians, THs are required for tadpole metamorphosis into a juvenile frog and disruption of TH action results in the inability of the

frog to develop normally (Shi 2000). Therefore, modification of TH action at early life history stages can lead to dramatic, deleterious outcomes for both humans and wildlife species. The mechanisms of TH action are common among vertebrates and involve a complex regulatory pathway yet the high degree of sensitivity to TH disruption of the tadpole has been identified as a powerful surrogate for the identification of chemicals and/or contaminants that perturb TH signaling in vertebrates (OECD 2009; Helbing 2012).

The invention presents an innovative method that relies upon a gene expression readout for detecting TH disrupting activities using Anuran species as model organisms, thereby greatly improving the power of the frog tadpole metamorphosis assay in predictivity and applicability to both laboratory testing and field applications across a range of all frog sentinel species. Moreover, the invention describes validated DNA primer sets for genes that are essential as references or “normalizers.” These normalizers can be used alone or in conjunction with primers used to ascertain TH responsiveness increasing the breadth of utility and application. Furthermore, this set of normalizer genes is not just specific for thyroid function, but will also serve as a strong suite of reference genes for any type of qPCR application utilizing any response genes evaluated for any study using Anuran species.

The invention comprises one or more DNA primer pairs (Table 1) that have been designed against “normalizer” and/or responsive genes such that the same primer pair representing a given gene can reliably and quantitatively function in Anura regardless of species.

TABLE 1

Sequence, qPCR running conditions and characteristics of the Anuran DNA primer set (Table 1 discloses SEQ ID NOS 1-30, respectively, in order of appearance)									
999 Gene	Gene Targets	Gene Abbreviation	Primer UP	Primer DOWN	Annealing Temperature	Thermocycle Profile	Amplification		
TAX 1	alpha 2 type 1 collagen	colla2	TAX1 up	CTGGTGGTGGG TATGATGGT	TAX1 dn	GAGTCTTAAGTC ACGGCAAG	60	15s, 30s, 45s	182
TAX 5	cytoplasmic beta- actin	actb	TAX5 up	TACAGCTTCACC ACCACAG	TAX5 dn	TCCACATCTGCT GGAAGGT	60/64*	15s, 30s, 45s	478
TAX 7	ribosomal protein S10	rps10	TAX7 up	TTTGCYTGGCGK CACTTTT	TAX7 dn	ARCRGCACTGCG YCTGTA	60	15s, 30s, 45s	213
TAX 8	myelin proteolipid protein A	plp1	TAX8a up	TGGCTGARGGATT YTAYACC	TAX8 dn	ACAGCAGAGCAG GCAAMGA	60	15s, 30s, 45s	256
TAX 9	thyroid hormone receptor alpha	thra	TAX9 up	TGATAAGGCCACA GGRTACCACTA	TAX9 dn	CGGGTGATCT TGTCGATRA	60	15s, 30s, 45s	141
TAX 10	thyroid hormone receptor beta	thrb	TAX10 up	CTATAGAAGAAAA CAGAGAAAARAGA	TAX10 dn	GAAGGCTTCTAA GTCCACTTTTCC	60	15s, 30s, 45s	237
TAX 12	deiodinase type II	dio2	TAX12a up	CCTGGCTCTSTAY GACTC	TAX12d dn	RGCTGATCCRA ARTTGAC	62	15s, 30s, 45s	295
TAX 15	TH-induced basic region leucine zipper containing	thibz	TAX15a up	ASCTCCRCAGAA YCAGCA	TAX15a dn	TCACGTACCAG GCCAAAA	62	15s, 30s, 30s	354

TABLE 1-continued

Sequence, qPCR running conditions and characteristics of the Anuran DNA primer set (Table 1 discloses SEQ ID NOS 1-30, respectively, in order of appearance)									
999 Gene	Gene Targets	Gene Abbre- viation		Primer UP		Primer DOWN	Annealing Tem- perature	Thermo- cycle Profile	Ampli- con
TAX 16	Kruppel- like factor 9	klf9	TAX16a up	CYGCTCAGTGT CTGGTGT	TAX16a dn	ARGGGCCGGTA CTTGT	62	15s, 30s, 30s	250
TAX 18	fibronectin	fn1	TAX18 up	GTTGCCATGARG GWGGAC	TAX18 dn	CCATTGCCWGT GCAGATA	60	15s, 30s, 45s	373
TAX 20	matrix metallo- peptidase 2	mmp2	TAX20a up	TACAACAGCTGC ACTGAT	TAX20a dn	TCCTTGTCATAGT CTTCTGT	62	15s, 30s, 30s	248
TAX 22	ribosomal protein L8	rpl8	TAX22 up	CAGGGGACAGA GAAAAGGTG	TAX22 dn	TGAGCTTTGTT GCCACAG	60	15s, 30s, 45s	270
TAX 23	elongation factor 1 alpha	eef1a1	TAX23 up	GCTGCTGGTGT GGTGART	TAX23 dn	AGCATGTTGTC ACCRITCC	60	15s, 30s, 45s	257
TAX 27	translocase of inner mitochondrial membrane 50 homolog	tim50	TAX27 up	GCTWCAYCCAGA GTGGTCGTT	TAX27 dn	GCTGYTCCTC CTGCTCCA	60	15s, 30s, 45s	497
TAX 28	ornithine trans carbamylase	otc	TAX28 up	YATGACYGATG CTGTTCTAG	TAX28 dn	CATAWCCCTTT GGTGTGTC	60	15s, 30s, 30s	272
*Xenopus laevis/Rana catesbeiana annealing temperatures									

\**Xenopus laevis*/*Rana catesbeiana* annealing temperatures

Examples of the use of the invention are to indicate responsiveness to thyroid hormone action or indicators of developmental phases attained. We demonstrate that these primers function well across the evolutionary range of Anurans and also demonstrate the range of tissues in which the primers may be reliably used in the context of assessing mRNA (cDNA) levels or gene levels (genomic DNA). Finally, we also present a specific application of the technology to identifying perturbations in thyroid hormone signaling.

A great diversity of Anuran species are used in a broad range of disciplines such as developmental and cancer biology, physiology, ecology, molecular biology, biochemistry, from egg to adult stages. Every qPCR-based study requires the use of reference gene transcripts or genes in order to be able to interpret experimental data. The basic assumption is that the reference gene(s) do not vary due to the treatment condition of the experiment and the type of reference or "normalizer" varies depending upon experimental context (Bustin, Beaulieu et al. 2010). Therefore, a selection of normalizer gene candidates is necessary as well as the identification and use of multiple normalizers to enhance the robustness of the data set. Furthermore, this set of normalizer genes is not just specific for thyroid function, but will also serve as a strong suite of reference genes for any type of qPCR application utilizing any response genes evaluated for any study using Anuran species.

The inventors have identified TH-responsive gene candidates that are common between mammals and frogs that would serve to act as meaningful indicators of TH disruption within the context of an amphibian metamorphosis assay (AMA) (Searcy, Beckstrom-Sternberg et al. 2012). The suggested species for the AMA is *Xenopus laevis* with additional interest in using the related *Silurana* (*Xenopus*)

*tropicalis* (Mitsui, Fujii et al. 2006). However, several nations are also interested in using native species relevant to their environments such as *Rana pipiens* and *Pseudacris regilla* (Canada), *Rana temporaria* (Europe), and *Rana rugosa* (Japan), *Rhinella* (*Bufo*) *marinus* (Latin America, Australia and SE Asia), or the only truly cosmopolitan frog, *Rana catesbeiana* (Bruno 2001; Veldhoen, Skirrow et al. 2006; Oka, Miyahara et al. 2009; Marlatt, Veldhoen et al. 2013). Toxicological evaluations on a variety of species is highly desirable to enable direct comparison between species for sensitivities and evaluation of the impact of different life histories and genome compositions (Relyea and Jones 2009; Helbing 2012).

The designed primers were subjected to tests of qPCR specificity and suitability within each tissue of interest in BOTH *Xenopus laevis* and *Rana catesbeiana*. These two species bracket the most commonly used frog families spanning ~200 million years of evolution (Sumida, Kato et al. 2004) (FIG. 1). The inventors determined that if the primer pairs work well for both species, then the likelihood that they work for intermediate species is assured.

FIG. 1 is an anuran cladogram depicting major families of current interest in the scientific and conservation realm. *Xenopus laevis* is a member of the Pipidae whereas *Rana catesbeiana* is a member of the Ranidae. Adapted from AmphibiaWeb: Phylogenetic view of Amphibia [web application] 2012. UC Regents, Berkeley, Calif., available: <http://amphibiaweb.org> (14 May 2012).

The inventors' rigorous design and verification protocol includes amplicon sequence confirmation, routine melting curve analysis, generation of standard curves for each primer set, and determination of amplification efficiency. All procedures are compliant with the Minimum Information for Publication of qPCR Experiments (MIQE précis) guidelines

(Bustin, Beaulieu et al. 2010). qPCR technology presents the most accurate and sensitive genetic technique available that enables high-throughput detection of low abundance mRNA transcripts. Due to its relative robustness and low operating cost, this technology is currently the most conducive to the demands of the regulatory context and the assays developed have immediate translation to regulatory labs. We elected to focus on a SYBR-based detection method because this is the presently least expensive to run (good for the price point of the end user) and more labs have access to the equipment necessary for readout therefore increasing the market potential.

Our primer quality assurance/control procedure has been standardized and follows a three-tiered quality control and quality assurance (QC/QA) process, as follows.

Tier 1 QC/QA Total RNA from the target animal species and tissue type is converted to complementary DNA (cDNA) and queried with the putative qPCR primer pairs designed against genes of interest. The function of each primer pair in the qPCR assay is assessed for amplification signal representative of a single targeted cDNA product. Control qPCR reactions containing no cDNA are performed to evaluate background inter-and intra-primer interactions that can contribute to signal noise. Thermocycle conditions are chosen for each gene-specific primer pair that maximizes signal specificity while minimizing background noise. The DNA products generated in the qPCR assay are subsequently confirmed to be the correct predicted length through agarose gel electrophoretic analysis.

Tier 2 QC/QA The specificity in DNA amplification of each qPCR primer pair is further confirmed through isolation and sequence-dependent assessment of qPCR-generated DNA products that contribute to the SYBR-based signal. Two methods can be used: 1) mapping of restriction endonuclease-derived fragments of qPCR amplified DNA, or 2) DNA sequencing of the product of qPCR. This serves to confirm the specificity of the qPCR SYBR-signal collected and the identity of the gene sequence amplified by each primer pair used in qPCR.

Tier 3 QC/QA To ensure that the SYBR-based signal data collected during qPCR can be employed in a quantitative manner, similarity in relative primer pair performance must be satisfied using information obtained through qPCR analysis of a dilution series of target cDNA from the tissue of interest. The sensitivity and ability of each gene-specific primer pair to detect linear changes in target cDNA abundance is assessed. Primer pair performance of a test gene is compared to the performance of primer pairs from reference genes to ensure comparable amplification efficiencies. Tissue context is important because we and others have found that some primers, although specific for their gene target, can display reduced performance in certain tissue contexts.

The results of the three tier QC/QA process for each of the primer sets listed in Table 1 are presented in FIG. 2. This demonstrates that the primer sets work to specifically amplify the appropriate amplicon in a variety of contexts. A summary of which tissue types are usable for transcriptomics analyses for each primer set is found in FIG. 8. In FIG. 8, the particular tissues of *Rana catesbeiana* and *Xenopus laevis* in which each primer set is usable are denoted as follows:

T=tail fin  
HL=hind limb  
I=intestine  
H=heart  
Li=liver  
B=brain  
Lu=lung

The QC/QA results plus evidence of functionality in *Rana catesbeiana* and *Xenopus laevis* tissues are presented in FIG. 2, which is comprised of FIGS. 2A(1) through FIGS. 2A(10); FIGS. 2B(1) through FIGS. 2B(10) and FIGS. 2C(1) through FIGS. 2C(10). In these figures, results of the three tier QC/QA process by primer set on tissues from *Rana catesbeiana* and *Xenopus laevis* are shown. Each of the 30 primer sets is identified at the top of each drawing figure. Quality assurance (QA) of each primer pair is established through a three-tier evaluation procedure that includes:

Tier 1, a clean cDNA-dependent signal return with low background noise determined through amplification that is associated with the production of a single DNA product for each anuran tissue type as determined through dissociation curves and electrophoretic analyses;

Tier 2, sequence-dependent confirmation of gene-specific targeting through restriction endonuclease mapping or direct DNA sequencing of the isolated amplified DNA product; and

Tier 3, primer pair comparative efficiency assessment across a tissue-specific cDNA dilution series as it related to the qPCR performance of the selected reference primer pair on the same cDNA dilution series. The slope of the interrelated qPCR performance must be  $\leq 0.1$  to be considered suitable for that tissue type.

The decision of whether a primer pair would be used to discern a gene transcript as a normalizer or test is highly dependent upon the experimental context and includes considerations of tissue-type, sample time, exposure type, and species examined.

Examples of normalizer gene candidates that would be used in any qPCR experiment to act as input reference include, but are not limited to: *coll1a2*, *actb*, *fn1*, *rps10*, *plp1*, *rpl8*, *eeef1a1* and *timm50* (Table 1).

Examples of thyroid hormone-responsive gene candidates in *Rana catesbeiana* (FIGS. 3 and 4) and *Xenopus laevis* (FIG. 5) include, but are not limited to: *coil a2*, *actb*, *plp1*, *thra*, *thrb*, *dio2*, *thibz*, *klf9*, *fn1*, *mmp2*, and etc.

FIG. 3 illustrates a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Rana catesbeiana*. Fold-change expression data for the indicated gene transcripts as determined using the comparative Ct method ( $\Delta\Delta Ct$ ) using multiple gene transcript normalizers. Data are expressed in box plots as fold-change in expression levels relative to the vehicle control group. Premetamorphic tadpoles were injected with either vehicle control (C) or the indicated concentration of thyroid hormone and the tissues collected after 48 hours. The concentration for  $T_3$  exposures are 0.01, 0.1, 1.0, 10, 25, and 50 nM and 0.05, 0.5, 5.0, 50, 125, and 250 nM for  $T_4$ . The medians are shown as solid black lines within the box and the box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Whiskers indicated the range. "a": significantly different from the vehicle control group ( $p < 0.05$ ). "n/a" means not expressed in that tissue.

FIG. 4 illustrates a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Rana catesbeiana*. Fold-change expression data for the indicated gene transcripts as determined using the comparative Ct method ( $\Delta\Delta Ct$ ) using multiple gene transcript normalizers. Data are expressed in box plots as fold-change in expression levels relative to the vehicle control group. Premetamorphic tadpoles were injected with either vehicle control (C) or the indicated concentration of thyroid hormone and the tissues collected after 48 hours. The concentration for  $T_3$  exposures are 0.01, 0.1, 1.0, 10, 25, and 50 nM and 0.05, 0.5, 5.0, 50, 125, and 250 nM for  $T_4$ . The medians are shown as solid

black lines within the box and the box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Whiskers indicated the range. “a”: significantly different from the vehicle control group (p<0.05). “n/a” means not expressed in that tissue.

FIG. 5 illustrates a demonstration of thyroid hormone responsiveness of the target gene transcripts in *Xenopus laevis* in the tail, brain, liver, and intestine. Fold-change

adults. For both species, complementary DNA (cDNA) was generated from total RNA isolated from mixed prometamorphic tadpole tissues. Location of the predicted PCR amplicon representative of expressed sequence for each gene target is shown by an arrowhead. MW denotes molecular size standards in kilobase pairs of linear DNA. The results for all of the primer sets are summarized in Table 3.

TABLE 3

Characterization of PCR amplified DNA products generated using genomic DNA of <i>Xenopus laevis</i> and <i>Rana catesbeiana</i> and the TAXISS primer panel <sup>a</sup> compared to amplicons generated from cDNA. The majority of primer sets amplify a distinctly different product or generate no product on genomic DNA from both species. This accentuates that, although the genomes of these species are very different, the ability to detect gDNA contamination in cDNA preparations is possible.							
TAXISS Primers	Gene Name	<i>Rana catesbeiana</i>			<i>Xenopus laevis</i>		
		Similar Product	Different Product	No Product	Similar Product	Different Product	No Product
TAX1	col1a2	+			+		
TAX5	actb			+		+	
TAX7	rps10		+			+	
TAX8	plp1			+		+	
TAX9	thra			+		+	
TAX10	thrb	+				+	
TAX12	dio2			+		+	
TAX15	thibz	+			+		
TAX16	klf9			+	+		
TAX18	fn1			+			+
TAX20	mmp2			+		+	
TAX22	rp18		+			+	
TAX23	eef1a1	+			+		
TAX27	timm50			+			+
TAX28	otc			+		+	

<sup>a</sup>PCR were performed using qPCR-associated reagents and thermoprofile conditions. Genomic DNA and cDNA reactions were carried out concurrently for comparison. The annealing temperature was 60° C. for all reactions.

<sup>b</sup>Relative comparison of amplicon band migration on agarose gels comparing amplicons generated from gDNA versus cDNA.

expression data for the indicated gene transcripts as determined using the comparative Ct method ( $\Delta\Delta C_t$ ) using multiple gene transcript normalizers. Data are expressed in box plots as fold-change in expression levels relative to the vehicle control group. Premetamorphic tadpoles were immersed in water containing vehicle control of 10 nM T<sub>3</sub> for 48 hours. The medians are shown as solid black lines within the box and the box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Whiskers indicate the range. Greyed out regions indicate no detectable specific signal in the indicated tissue (refer to FIG. 8 for details). “a”: significantly different from the vehicle control group (p<0.05).

The primer sets can be used on multiple Anuran species beyond *Rana catesbeiana* and *Xenopus laevis*, as is demonstrated in FIG. 6. FIG. 6 illustrates a demonstration that the primer sets are usable in a wide range of anuran species. The primer sets in Table 1 were run on cDNA preparations made from a single tissue or mixture of tissues (species-dependent) on the indicated species to demonstrate that the appropriately sized amplicon is produced across a wide range of Anuran species.

Another use of the primers can be for detection of contaminating genomic DNA in transcriptomic studies or in amplification of genomic DNA (FIG. 7) and their utility in *Rana catesbeiana* and *Xenopus laevis* are summarized in Table 3. FIG. 7 illustrates representative examples of primer set performance on genomic DNA across different anuran species. Genomic DNA (gDNA) was isolated from skin of *Xenopus laevis* tadpoles and liver of *Rana catesbeiana*

Each primer pair can be used independently of each other. In one embodiment, the invention comprises use of a primer pair by itself to detect particular genes within an Anuran nucleic acid sample.

In another embodiment, two or more of any of the primer pairs listed in Table 1 may be used.

The normalizer gene panel would include two or more of the following primer pairs: actb, rps10, rp18, and eef1a1. The end-user would then be able to use evaluation tools readily available to finalize the appropriateness of each reference gene.

The thyroid hormone responsive gene panel would include two or more of the following primer pairs: colla2, plp1, thra, thrb, dio2, thibz, klf9, fn1, mmp2, otc and timm50.

The assay of the invention further comprises the following method. Tadpoles are exposed to the compound of interest in water and, at the appropriate time (e.g., within hours to a few days), two standardized portions of the tail are removed. The tail tips are immediately subjected to an apoptosis assay in Phase 1, while the other (second) tail portion is preserved for Phase 2.

If the results for Phase 1 are negative, then the substance is not thyroid axis active. No further analysis is necessary. If the Phase 1 results are positive, then the second tail portion is analyzed for shifts in the quantity of mRNA transcripts specifically identified for responsiveness in Phase 2. An example of Phase 2 analysis is using quantitative real time polymerase chain reaction (qPCR) for each gene transcript target.

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If the gene transcripts indicative of thyroid hormone (TH) action are not affected, then it can be concluded that the test compound is not thyroid axis active. If the transcripts are affected, then the compound is identified as a likely TH disruptor.

Accordingly, disclosed herein is a frog tadpole metamorphosis assay method comprising a first phase and a second phase, wherein:

the first phase comprises exposing a tadpole to a compound of interest, removing first and second portions of the tadpole's tail, subjecting the first portion to an apoptosis assay, and

the second phase comprises subjecting the second portion to an assay to detect shifts in a quantity of pre-determined mRNA transcripts.

In a more specific embodiment of the method, the first phase further comprises determining whether the compound of interest is thyroid axis active.

In yet another embodiment of the method, the second phase further comprises subjecting the second portion to qPCR.

In still yet another embodiment, the the first phase further comprises determining whether the compound of interest is thyroid axis active, and the second phase further comprises subjecting the second portion to qPCR.

In an embodiment of the invention, disclosed is a metamorphosis assay wherein the first phase comprises exposing a tadpole to a compound of interest, removing first and second portions of the tadpole's tail, and subjecting the first portion to an apoptosis assay, and the second phase comprises subjecting the second portion to qPCR using a primer pair comprising a first primer and a second primer, wherein the primer pair is selected from the group consisting of primer pairs A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
A	CTGGTGGTGG ATATGATGGT	1	GAGTCTTAAG TCACGGCAAG	2
B	TACAGCTTCA CCACCACAG	3	TCCACATCTG CTGGAAGGT	4
C	TTTGCYTGGC GKCACTTTT	5	ARCRGCACTG CGYCTGTA	6
D	TGGCTGARGG ATTYTAYACC	7	ACAGCAGAGC AGGCAAMGA	8
E	TGATAAGGCC ACAGGRTACC ACTA	9	CGGGTGATCT TGTCGATRA	10
F	CTCATAGAAG AAAACAGAGA AAARAGA	11	GAAGGCTTCT AAGTCCACTT TTCC	12
G	CCTGGCTCTS TAYGACTC	13	RGCTGATCCR AARTTGAC	14
H	ASCTCCRCAG AAYCAGCA	15	TCACGTACCA GGCCAAAA	16
I	CYGCTCAGTG TCTGGTGT	17	ARGGGCCGGT ACTTGTTT	18
J	GTTGCCATGA RGWGGAC	19	CCATTGCCWG TGCAGATA	20
K	TACAACAGCT GCACTGAT	21	TCCTTGTCAT AGTCTTCTGT	22

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-continued

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
L	CAGGGGACAG AGAAAAGGTG	23	TGAGCTTTCT TGCCACAG	24
M	GCTGCTGGTG TTGGTGART	25	AGCATGTTGT CACCRTTCC	26
N	GCTWCAYCCA GAGTGGTCGT T	27	GCTGYTCCTC CTGCTCCA	28
O	YATGACYGAT GCTGTTCTAG	29	CATAWCCCTT TGGTGTTCG	30

In yet another particular embodiment of the invention, the second phase further comprises subjecting the second portion to qPCR using two or more primer pairs, wherein each pair comprises a first primer and a second primer, and the primer pairs are selected from the group consisting of primer pairs A, B, D, E, F, G, H, I, J, K, and O:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
A	CTGGTGGTGG ATATGATGGT	1	GAGTCTTAAG TCACGGCAAG	2
B	TACAGCTTCA CCACCACAG	3	TCCACATCTG CTGGAAGGT	4
D	TGGCTGARGG ATTYTAYACC	7	ACAGCAGAGC AGGCAAMGA	8
E	TGATAAGGCC ACAGGRTACC ACTA	9	CGGGTGATCT TGTCGATRA	10
F	CTCATAGAAG AAAACAGAGA AAARAGA	11	GAAGGCTTCT AAGTCCACTT TTCC	12
G	CCTGGCTCTS TAYGACTC	13	RGCTGATCCR AARTTGAC	14
H	ASCTCCRCAG AAYCAGCA	15	TCACGTACCA GGCCAAAA	16
I	CYGCTCAGTG TCTGGTGT	17	ARGGGCCGGT ACTTGTTT	18
J	GTTGCCATGA RGWGGAC	19	CCATTGCCWG TGCAGATA	20
K	TACAACAGCT GCACTGAT	21	TCCTTGTCAT AGTCTTCTGT	22
O	YATGACYGAT GCTGTTCTAG	29	CATAWCCCTT TGGTGTTCG	30

The invention further relates to a composition of matter comprising a primer pair selected from the group consisting of primer pairs A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
A	CTGGTGGTGG ATATGATGGT	1	GAGTCTTAAG TCACGGCAAG	2

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-continued

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
B	TACAGCTTCA CCACCACAG	3	TCCACATCTG CTGGAAGGT	4
C	TTTGCYTGGC GKCACTTTT	5	ARCRGCACTG CGYCTGTA	6
D	TGGCTGARGG ATTYTAYACC	7	ACAGCAGAGC AGGCAAMGA	8
E	TGATAAGGCC ACAGGRTACC ACTA	9	CGGGTGATCT TGTCGATRA	10
F	CTCATAGAAG AAAACAGAGA AAARAGA	11	GAAGGCTTCT AAGTCCACTT TTCC	12
G	CCTGGCTCTS TAYGACTC	13	RGCTGATCCR AARTTGAC	14
H	ASCTCCRCAG AAYCAGCA	15	TCACGTACCA GGCCAAA	16
I	CYGCTCAGTG TCTGGTGT	17	ARGGGCCGGT ACTTGT	18
J	GTTGCCATGA RGGWGGAC	19	CCATTGCCWG TGCAGATA	20
K	TACAACAGCT GCACTGAT	21	TCCTTGTCAT AGTCTTCTGT	22
L	CAGGGGACAG AGAAAAGGTG	23	TGAGCTTTCT TGCCACAG	24
M	GCTGCTGGTG TTGGTGART	25	AGCATGTTGT CACCRITCC	26
N	GCTWCAYCCA GAGTGGTCGT T	27	GCTGYTCCTC CTGCTCCA	28
O	YATGACYGAT GCTGTTCTAG	29	CATAWCCCTT TGGTGTTCG	30

Yet another aspect of the invention relates to a normalizer gene panel comprising two or more primer pairs selected from the group consisting of primer pairs A, B, C, D, L, M, and N:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
A	CTGGTGGTGG ATATGATGGT	1	GAGTCTTAAG TCACGGCAAG	2
B	TACAGCTTCA CCACCACAG	3	TCCACATCTG CTGGAAGGT	4
C	TTTGCYTGGC GKCACTTTT	5	ARCRGCACTG CGYCTGTA	6
D	TGGCTGARGG ATTYTAYACC	7	ACAGCAGAGC AGGCAAMGA	8
L	CAGGGGACAG AGAAAAGGTG	23	TGAGCTTTCT TGCCACAG	24
M	GCTGCTGGTG TTGGTGART	25	AGCATGTTGT CACCRITCC	26
N	GCTWCAYCCA GAGTGGTCGT T	27	GCTGYTCCTC CTGCTCCA	28

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Yet another aspect of the invention relates to a thyroid hormone responsive gene panel comprising two or more primer pairs selected from the group consisting of primer pairs A, B, D, E, F, G, H, I, J, K, and O:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
A	CTGGTGGTGG ATATGATGGT	1	GAGTCTTAAG TCACGGCAAG	2
B	TACAGCTTCA CCACCACAG	3	TCCACATCTG CTGGAAGGT	4
D	TGGCTGARGG ATTYTAYACC	7	ACAGCAGAGC AGGCAAMGA	8
E	TGATAAGGCC ACAGGRTACC ACTA	9	CGGGTGATCT TGTCGATRA	10
F	CTCATAGAAG AAAACAGAGA AAARAGA	11	GAAGGCTTCT AAGTCCACTT TTCC	12
G	CCTGGCTCTS TAYGACTC	13	RGCTGATCCR AARTTGAC	14
H	ASCTCCRCAG AAYCAGCA	15	TCACGTACCA GGCCAAA	16
I	CYGCTCAGTG TCTGGTGT	17	ARGGGCCGGT ACTTGT	18
J	GTTGCCATGA RGGWGGAC	19	CCATTGCCWG TGCAGATA	20
K	TACAACAGCT GCACTGAT	21	TCCTTGTCAT AGTCTTCTGT	22
O	YATGACYGAT GCTGTTCTAG	29	CATAWCCCTT TGGTGTTCG	30

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## SEQUENCE LISTING

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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    primer

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<400> SEQUENCE: 24

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21

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&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

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18

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&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

&lt;400&gt; SEQUENCE: 29

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&lt;210&gt; SEQ ID NO 30

&lt;211&gt; LENGTH: 19

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

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&lt;400&gt; SEQUENCE: 30

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19

The invention claimed is:

1. A frog tadpole metamorphosis assay method comprising a first phase and a second phase, wherein:

the first phase comprises exposing a tadpole to a compound of interest, removing first and second portions of the tadpole's tail, subjecting the first portion to an apoptosis assay, and

the second phase comprises subjecting the second portion to an assay to detect shifts in a quantity of pre-determined mRNA transcripts, wherein the second phase further comprises subjecting the second portion to qPCR using a primer pair comprising a first primer and a second primer, wherein the primer pair is selected from the group consisting of primer pairs B, C, L, M:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
B	TACAGCTTCACCAACACAG	3	TCCACATCTGCTGG AAGGT	4
C	TTTGCTGGCGKCACTTTT	5	ARCRGCACTGCGYC TGTA	6
L	CAGGGGACAGAGAAAAGG TG	23	TGAGCTTTCTTGCC ACAG	24
M	GCTGCTGGTGTGGTGART	25	AGCATGTTGTCACC RTTCC	26

2. The assay method of claim 1, wherein the first phase further comprises determining whether the compound of interest is thyroid axis active.

3. The assay method of claim 1, wherein the second phase further comprises subjecting the second portion to qPCR using two or more primer pairs.

4. A composition of matter comprising a primer pair selected from the group consisting of primer pairs B, C, L, and M:

Primer Pair	First Primer	SEQ ID NO:	Second Primer	SEQ ID NO:
B	TACAGCTTCACCAACACAG	3	TCCACATCTGCTGG AAGGT	4
C	TTTGCTGGCGKCACTTTT	5	ARCRGCACTGCGYC TGTA	6
L	CAGGGGACAGAGAAAAGG TG	23	TGAGCTTTCTTGCC ACAG	24
M	GCTGCTGGTGTGGTGART	25	AGCATGTTGTCACC RTTCC	26

\* \* \* \* \*